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CHLORINE, BROMINE AND SODIUM AS NUTRIENTS FOR SUGAR BEET PLANTS[1]

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Chlorine, sodium and silicon have long been considered as elements that are possibly essential to the growth and development of sugar beets and other plants. Of these elements Cl has been found just recently by Broyer et al (2) to correct a severe nutritional deficiency of the tomato plant when grown in low halide culture solutions. Raleigh (28) observed enhanced growth of table beets in culture solutions supplied with NaCl instead of Na_2SO_4 as the source of Na. However, no symptoms attributable to a Cl deficiency were reported by him. Crowther (6) noted that sugar beet plants wilted less when Na was applied as NaCl than when supplied as Na_2SO_4. Significant responses to chloride additions were observed in nutrient solutions by Lipman (21) for buckwheat, by Eaton (7) for tomatoes and cotton, and by Kretschmer et al (16) for lima bean fruit.

As for Br, it has not been considered as a nutrient favorable to plant growth, even though it has been used repeatedly in ion absorption studies by many investigators for many years.

Much has been written about Na in terms of direct and indirect effects upon plant growth (5, 9, 10, 19, 20, 23). As an indirect factor of growth the beneficial effects of Na applications to the soil have been explained frequently as a release of K from the soil, or as promoting better root development (5). These phenomena have been considered of special importance in meeting the K requirements of plants on soils low in K. Quite often too the growth of plants has been increased by the addition of Na salts to soils (4, 5, 8, 17, 36) or to nutrient solutions low in K (12, 24, 35). When plants are high in K, Na responses have been observed (12, 18, 31) but most often these are much reduced or not at all in evidence (4, 17, 18, 22, 35). Direct effects of Na on sugar beet growth in field experiments have been reported for muck soils by Harmer and Benne (9) and Harmer et al (10), for sand cultures by Tullin (32), and for table beets in pot experiments with soils by Larson and Pierre (18). Sodium deficiency symptoms have been reported (32) or described (9) in only a few instances and so far no specific function of Na, not performed by K, has been recorded (17, 19). Yet for the beet, "sodium may almost be deemed an indispensable nutrient element, approaching potassium in importance" (19).

Silicon, when added as a silicate to soils, has been considered primarily as contributing to the phosphate supply of the soil (29). Raleigh (26, 27) reported increased growth of table beets when silicates were added to culture solutions. He was unable to conclude from his work whether the favorable effect was due to a healthier root system or to a specific need for Si by the plants for growth.

In view of these reports, often indefinite in their conclusions, it was thought worthwhile to re-examine Na, Si and Cl in relation to the growth of sugar beet plants by adding these elements to otherwise complete culture solutions prepared from "chemically pure" salts. The initial experiment was of a factorial design using two levels of the elements in question. The first level of Cl, Na or Si was the amount present as an impurity in the salts and distilled water. The other level was an exact quantity added. It was thought that if the requirement for any one or more of these elements was larger than that provided by the impurities present, it would be revealed by the increased growth of the plants at the higher nutrient level. If increased growth was not obtained, the second step to be taken would include a further purification of all salts for each of the elements not found to enhance growth or development of the sugar beet plant. Two other factors were also included in the experiment; K at 2 levels and 2 beet varieties, giving a total of 5 factors each at 2 levels.

METHODS AND MATERIALS

PLANT CULTURE: The sugar beet (*Beta vulgaris*) varieties used in the 2^5 factorial experiment were U.S. 22/3 and GW 304, and for the chloride series experiment, U.S. 75. In each instance 25 gm of seed were treated with 0.25 gm of the fungicide Phygon XL (United States Rubber Company, Naugatuck Chemical Division, New York, New York). These seeds were planted 2.0 cm deep in a $31 \times 76 \times 20$ cm germinating tray filled with industrial No. 2 vermiculite (California Zonolite Company, San Francisco, California). The seeds and seedlings were watered daily with one-half strength Hoagland's culture solution (11). When the seedlings were in the early 2-leaf stage, single plants were transplanted to cork rings having approximately 6.0 cm outside and 4.0 cm inside diameters. The plants were held in place with non-absorbent cotton. Three plants prepared in this way were taken at random and inserted into a mason-

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ite cover for each culture solution tank. The tanks were standard black iron buckets of 20-l capacity that were lined on the inside with a plastic coating, Amercoat No. 33 (Amercoat Corp., South Gate, California) and on the outside with aluminum paint. All culture solutions were aerated continuously.

The compositions of the culture solutions for the 2^5 factorial experiment are given in table I. The treatments essentially involved 2 sugar beet varieties and 2 levels of Na, Cl, Si and K, giving 32 treatments in duplicate or a total of 64 separate 20-l tanks for the experiments. The plants were set out on October 12, 1953 and harvested on January 14, 1954.

The salts for the chloride series experiment were of the following initial concentrations: 0.001 M KH_2PO_4, 0.00375 M $Ca(NO_3)_2$, 0.001 M $MgSO_4$, 0.00025 M Na_2SiO_3, 0.25 ppm B, 0.25 ppm Mn, 0.025 ppm Zn, 0.01 ppm Cu, 0.005 ppm Mo. Iron was added at the rate of 2.5 ppm as the ferric potassium ethylene diamine tetra-acetate complex (13). Salts equivalent to the above concentrations were added on June 3, and again on July 14, 1954. Chlorine as KCl, and bromine as KBr were added one time only in the concentrations outlined in table IV. Potassium sulfate (0.5 M) was used to equalize the K concentrations for the various Cl levels. The culture solutions for both experiments were prepared from salts of the analytical grade that tested low in halide. No special purification relative to Cl or Br was found necessary to produce halide deficiency symptoms on sugar beet plants. Except for the salt additions as noted, the solutions were not changed during the course of the experiment. The plants were grown from June 8 to August 2, 1954.

HARVEST: The tops of the plants were separated from the roots at the first leaf scar. The fibrous roots were removed from the storage root, centrifuged for 5 minutes at 39 × g, weighed, and dried at 80° C in a forced draft oven. The storage roots were weighed and pulped, and 3 samples weighing 26.0 gm each were frozen with dry ice immediately and analyzed for sucrose at a later date. The tops of the plants were separated into recently matured leaves, i.e., those normally showing the first symptoms of K deficiency, and immature leaves, i.e., those which normally show the first Cl deficiency symptoms. The petioles and blades of these leaves were weighed and dried separately.

In the Cl series experiment the leaf separations were extended to include the living leaves older than the ones matured recently. Only the upper half of each blade, exclusive of the mid-rib, was saved as the "blade" material for chemical analysis. The petiole material did not include the mid-rib of the leaf blade. All leaf material after drying at 70 to 80° C was ground to pass the 40-mesh sieve of a Wiley mill.

ANALYTICAL PROCEDURE, *Chlorine:* The very small amounts of Cl in the plant material of Cl deficient plants made it necessary to adopt the micro-diffusion method for Cl as described by Conway (3). This method was modified for plant material and is based on that used by Broyer et al (2). Ground plant material (0.5 gm for Cl deficient and 0.1 gm for Cl treated plants) was mixed thoroughly with a low halide CaO which was prepared as recommended by Piper (25). The CaO-sample mixture was moistened with water, allowed to stand for 30 minutes, evaporated to dryness and ashed for 15 hours at 500° C in an electric muffle. The ash was dissolved by adding 5.0 ml redistilled water and 5.0 ml of 4.0 *N* HNO_3 and then digesting on the steambath for 5 minutes. The extract was made up to a 25.0 ml volume. An appropriate aliquot of this extract was placed in the outer compartment of a Conway dish and the total sample volume was made up to 2.0 ml with redistilled water. Three ml of freshly prepared 6.7 % KI was added to the center compartment. An oxidant mixture of 30 % H_2SO_4 and 3.0 % $KMnO_4$ (prepared by mixing 15.0 ml of precooled 60 % H_2SO_4 and 15.0 ml of 6 % $KMnO_4$ in an ice bath) was added to the outer

TABLE I

CHEMICAL COMPOSITION OF NUTRIENT SOLUTIONS IN MILLIEQUIVALENTS PER LITER FOR 2^5 FACTORIAL EXPERIMENT *

TREATMENTS	HIGH POTASSIUM SOLUTIONS						LOW POTASSIUM SOLUTIONS					
	K^+	Ca^{++}	Na^+	$SO_4^=$	Cl^-	$SiO_3^=$	K^+	Ca^{++}	Na^+	$SO_4^=$	Cl^-	$SiO_3^=$
0	4.0	5.0	0	2.25	0	0	0.5	8.5	0	2.25	0	0
Cl	4.0	5.5	0	2.25	0.5	0	0.5	8.5	0	2.25	0.5	0
Na	4.0	5.0	0.5	2.75	0	0	0.5	8.5	0.5	2.75	0	0
NaCl	4.0	5.0	0.5	2.25	0.5	0	0.5	8.5	0.5	2.25	0.5	0
Si	4.0	5.0	0	2.25	0	0.5	0.5	8.5	0	2.25	0	0.5
SiCl	4.0	5.5	0	2.25	0.5	0.5	0.5	9.0	0	2.25	0.5	0.5
NaSi	4.0	5.0	0.5	2.25	0	0.5	0.5	8.5	0.5	2.25	0	0.5
NaSiCl	4.0	5.0	0.5	2.25	0.5	0.5	0.5	8.5	0.5	2.25	0.5	0.5

* Both the high and low K solutions also received 7.5 meq NO_3^-, 1.0 meq $H_2PO_4^-$, 2.0 meq Mg^{++}, 0.25 mg Mn^{++}, 0.25 mg B, 0.025 mg Zn^{++}, 0.01 mg Cu^{++}, 0.005 mg Mo and 4.9 mg Fe^{+++} per liter. The iron was added as the ferric potassium ethylene diamine tetra-acetate complex, prepared as recommended by Jacobson (13). Silicon was added either as H_2SiO_3 (suspension) or as Na_2SiO_3 (solution).

Salts equivalent to the foregoing concentrations were added to the 20-l tanks on October 9, 1953 and again on November 24, 1953. Through these additions the low-K and high-K plants received a total of 20 and 160 meq K per 20-l tank, respectively. An extra 0.5 mg Mn^{++}/l was added as $MnSO_4$ to all solutions on December 11, 1953.

compartment. A pre-greased glass lid was placed on the Conway dish immediately after the addition of the oxidant. The samples were covered with a white towel and left on the laboratory bench for 2 hours. At the end of this time the glass lids were removed and the KI solution in the center compartment was transferred by suction to a 5.0-ml volumetric flask. During the transfers, the center compartment was thoroughly rinsed with a 0.4 % starch solution. The flasks were made up to a 5.0-ml volume with a 0.4 % starch solution (3). The absorbance of the starch iodide color complex was determined by a Beckman Model B spectrophotometer with a λ of 540 mμ.

Bromine: The Br analyses were done in 2 parts, 1) an analysis of total halide and 2) an analysis of Br. The difference was assumed to be the Cl content. The analysis of the total halide was similar to the method for Cl analysis with the following exceptions: 1) three ml of a solution of 0.6 % starch and 3.33 % KI was aded to the center compartment, 2) the Conway dishes were shaken gently at 60 cycles per minute for 1.5 hours during the oxidation period, 3) during the transfers the center compartment was rinsed with redistilled water. The analytical procedure for bromine was essentially the same as for the total halide with the following change: 1.0 ml of 40 % K_2CrO_4 was added to the outer compartment followed immediately by 1.0 ml of 80 % H_2SO_4.

Sucrose: The sucrose concentration of the beet roots was estimated in a saccharimeter after preparing a hot water extract of the frozen beet pulp samples as described by Browne and Zerban (1).

Other Elements: Potassium and Na were determined with a Perkin-Elmer flame photometer; NO_3-N by the phenol-disulphonic acid method (15); PO_4-P soluble in 2 % acetic acid by the phospho-molybdate method (33); and SO_4-S by the H_2S-methylene blue procedure (14).

Results

2^5 Factorial: The sugar beet plants for the 2^5 factorial experiment were transplanted at the 2-leaf stage on Oct. 12, 1953 and harvested Jan. 14, 1954. The plants grew exceedingly well at the high K level except for a minor chlorosis of the blades of the immature and recently matured leaves of some of the plants. The symptoms were first observed on December 10, 1953 and were particularly noticeable when the leaves were viewed through transmitted light. At that time the symptoms were thought to be an incipient Mn deficiency which had appeared even though Mn had been added to all nutrient solutions. On December 11 an attempt was made to correct the supposed deficiency by adding 0.5 ppm Mn as $MnSO_4$, to all cultures. However, one week later the symptoms still persisted. A review of all plants in the experiment was made on December 23. This inspection revealed that of the 32 cultures high in K, regardless of variety or the presence or absence of Na or Si, only the plants in the 16 cultures without Cl added showed the symptoms (fig 1). In the 32 cultures low

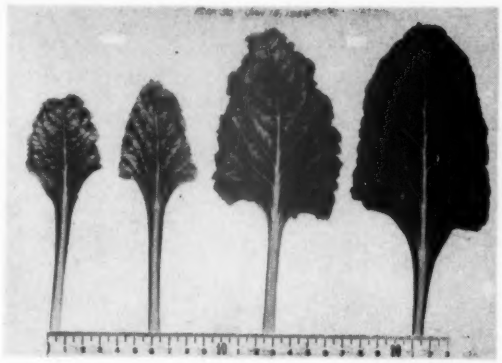

Fig. 1. Blades of sugar beet leaves from Cl deficient plants (*3 leaves on the left*) and from a non-deficient plant (*leaf on the right*).

in K all plants showed the usual K deficiency symptoms on the recently matured leaves, but in addition the center leaves of the plants in 15 out of the 16 cultures without the Cl added showed the same symptoms as the high K plants that were lacking Cl. Thus, the K deficient plants without Cl added showed simultaneously the usual K deficiency symptoms on the recently matured leaves and the Cl deficiency symptoms on the immature center leaves of the sugar beet plant. The immature leaves of the plants low in K but with the Cl added were normal and without symptoms.

The symptoms of Cl deficiency appear first as a chlorosis on the leaf blades of the younger leaves of the plant. The main veins and even the minor veins of these leaves remain green, while the interveinal areas are a light green to yellow in color. Through transmitted light, the leaf blade has a netted mosaic pattern with the branches of the veins forming the netting. Early phases of this pattern are reminiscent of Mn deficiency on sugar beet leaves. As the symptoms develop, the interveinal areas appear as smooth flat depressions, light green to yellow in color, which is in striking contrast to the green veins having a "raised" appearance. The advanced stages of the deficiency for the sugar beet plant are unique for Cl and are clearly distinguishable from the mottling as expressed by Mn, Fe, or Mo deficiency. Another special feature of Cl deficiency for plants grown in culture solution is the very stubby growth of the roots. The many stubs arising from secondary roots form a distinctive and abnormal root structure (fig 2).

In addition to the Cl deficiency symptoms noted on the leaves and roots of the sugar beet plant, the growth of the plant was retarded. These effects of Cl and those of Na, Si, and K are presented in table II as average values for the two sugar beet varieties studied. From the average values and from the F-values of the statistical analyses (30) it is evident that the addition of Cl as the Ca or Na salt (table I) to low Cl culture solutions has resulted in significant increases in the fresh and dry weights of tops and

fibrous roots for plants at both K levels (table II). However, the addition of Cl to high K plants did not increase the size of the storage root. Apparently the time interval prior to harvest was insufficient for the larger tops to effect an increase in root size. In the low K plants adding Cl actually decreased the storage root size instead of increasing it as in the fibrous roots.

The effects of Cl upon the sucrose concentration of the beet roots depend upon the K status of the plants. When plants are high in K, a Cl deficiency causes a decrease in the sucrose concentration of the beet roots, but when the plants are low in K, a Cl deficiency increases the sucrose concentration of the beet roots. This phenomenon can account for the conflicting reports of the effects of K deficiency on sucrose concentration. Thus, when large amounts of Cl are present in the culture solution as an impurity, a K deficiency decreases the sucrose concentration of the beet root but when Cl is low or deficient, a K deficiency increases the sucrose concentration of the beet root.

The Cl concentrations of the petioles of immature and recently matured leaves of Cl deficient plants (table III) are the same as those at the lowest Cl level in the Cl series experiment to be discussed later (see table IV). These values (table III) are approximately the same for both the high and low K plants. Neither the addition of Na, which increased the growth of the low K plants, nor that of Si influenced the petiole Cl concentrations of the plants deficient in Cl. The Cl concentrations of the high Cl-low K plants were much higher than for the high Cl-high K plants. This decrease in Cl concentration is a matter of dilution by growth rather than an interference of Cl absorption by plants high in K. Sodium added to the high Cl plants, except for the Si and NaSi comparison increased the Cl concentration of the petioles regardless of the K level; the increases being more pronounced for the mature than for the immature petioles (table III).

An inspection of the top growth made by the plants at the 2 K and Cl levels reveals an interesting interplay of these 2 elements (table II). Growth of the low K-low Cl plants can be increased for example, from 230 to 343 gm/pot by the addition of Cl, or from 230 to 500 gm/pot by the addition of K. In the presence of adequate amounts of Cl, the addition of K to the low K culture increased the growth from 343 to 693 gm/pot. At first glance, the increase in growth from 230 to 343 gm/pot by adding Cl to the low K culture appears to have extended the effectiveness of K in a manner analogous to the recognized sparing action of Na for low K plants (comparison of Cl, 343, with NaCl, 415). However, the effect of Cl added to the low K plants is not the same as that of Na, since

FIG. 2. Roots of Cl deficient (*upper, left*) and of non-deficient (*upper, right*) sugar beet plants. Roots in center photograph are from Cl deficient plants and those in lower photograph from non-deficient plants. Roots have been set into 4-l beakers for photographing only.

TABLE II

EFFECTS OF CHLORINE, POTASSIUM, SODIUM AND SILICON UPON SUGAR BEET PLANTS

TREATMENT *	TOPS				ROOTS				SUCROSE %		BLADE K %	
	FRESH GM		DRY GM		STORAGE, FRESH GM		FIBROUS, DRY GM					
	HIGH K	LOW K	HIGH K	LOW K	HIGH K	LOW K	HIGH K	LOW K	HIGH K	LOW K	HIGH K	LOW K
					Treatment × K means (4 values)							
0	500	230	52.7	32.5	145	68	2.05	2.10	8.4	10.4	6.42	0.58
Cl	693	343	65.2	38.2	166	43	2.72	4.30	9.3	8.9	5.18	0.57
Na	527	277	58.3	39.9	155	89	3.25	2.55	8.0	9.7	5.37	0.43
NaCl	744	415	72.9	43.9	195	59	4.23	4.76	9.3	9.2	3.91	0.57
Si	529	220	59.1	29.7	174	65	2.88	2.02	9.1	9.8	5.57	0.63
SiCl	735	329	72.1	37.1	191	53	3.71	4.87	10.4	8.2	4.22	0.64
NaSi	537	314	61.0	44.6	216	103	2.49	3.26	8.9	9.1	4.65	0.50
NaSiCl	833	444	84.7	49.5	225	69	6.09	6.27	10.4	9.2	3.55	0.57
LSD (Student) 5 % level	116	74	10.6	7.9	n.s.	21	1.33	1.22	n.s.	n.s.	1.13	n.s.
LSD (Student) 1 % level	160	102	14.6	10.9	n.s.	29	1.83	1.68	n.s.	n.s.	1.55	n.s.
					Variety × K means (16 values)							
U.S. 22/3	608	298	60.5	36.1	175	69	2.94	3.17	8.9	9.4	5.02	0.55
GW 304	667	345	71.0	42.8	191	69	3.91	4.36	9.5	9.2	4.69	0.57
					F-values **							
Cl	69.3	48.9	40.8	8.8	2.0	25.8	23.5	79.5	8.4	10.6	23.4	2.1
Si	2.4	0.4	7.8	0.7	5.7	2.6	5.4	5.6	4.8	2.8	7.4	1.6
Na	2.8	21.9	7.8	29.4	3.7	20.7	14.1	9.6	0.1	0.0	13.6	5.3
Variety	4.7	7.1	17.6	13.2	1.1	0.0	9.6	17.0	1.9	0.3	1.5	0.1

* Cl, Na, Si refer to the high level treatment.

** Required F-values for the one degree of freedom for each treatment, Cl, Si, Na, and Variety, and the 16 degrees of freedom for error are 4.49 and 8.53 for the 5 % and 1 % levels, respectively. The treatment interactions were not significant statistically, except for a few instances at the 5 % level only.

the distinctive Cl deficiency symptoms in the center leaves disappear upon the addition of Cl. A probable explanation for the response by adding either Cl or K to low Cl-low K plants can be found in the different abilities of these 2 ions to be reutilized within the plant. Potassium is readily mobilized from older leaves and moved to newly formed ones, whereas with Cl this does not appear to be the case. Thus, for plants deficient in both K and Cl, added Cl removes the Cl stress of the newly formed center leaves, and the Cl deficiency symptoms disappear. With the Cl stress removed, the younger center leaves utilize the K from the older leaves and more plant growth takes place. When K_2SO_4 was added to low Cl-low K plants Cl (being an impurity in this salt) was added also. Since the quantitative requirement for Cl is considerably lower than for K, both deficiencies were corrected together for a time and extra growth resulted until Cl again became deficient. The Cl added as an impurity from the K_2SO_4 in this experiment was, however, insufficient for continuous growth for high K plants.

The significant effects of Si on the growth of the sugar beet plant are not easy to assess from the results of the present experiment. None of the effects attained the 1 % level of significance but at the 5 % level the effects of Si occurred mainly with the plants high in K. Thus, on the average, the presence of Si, either as the Na salt or as silicic acid increased the dry weight of the tops, the fresh weight of the fibrous roots, and the sucrose concentrations of the beet roots. In the low K plants only the weights of the fibrous roots were significantly increased by the addition of Si to the culture solution.

In accord with the findings of other investigators, the addition of Na to low K plants increased significantly the weight of the tops and the weight of the storage and fibrous roots (table II). The sucrose concentrations of the storage roots were not changed appreciably by the Na treatments for either the low or high K plants. For the plants high in K, the addition of Na increased the dry weight of the tops and the dry weight of the fibrous roots but had no significant effect upon the weights of the fresh tops or upon the beet roots.

The GW 304 variety made significantly better top growth and had more fibrous roots than the U.S. 22/3, when grown at either K level (table II). Neither variety, however, differed significantly in beet root weight or in sucrose concentration. Nor were there

TABLE III

PETIOLE CHLORIDE (DRY BASIS) AS AFFECTED BY AGE OF LEAF AND POTASSIUM, SODIUM, SILICON AND CHLORINE NUTRITION

TREATMENT *	YOUNG PETIOLES		MATURE PETIOLES	
	HIGH Cl PLANTS μEQ/GM	LOW Cl PLANTS μEQ/GM	HIGH Cl PLANTS μEQ/GM	LOW Cl PLANTS μEQ/GM
Treatment × age × Cl means (4 values)				
0	410	7.3	444	9.9
K	133	6.7	305	8.9
Si	483	7.9	528	11.7
SiK	141	5.8	300	7.2
Na	457	6.1	558	9.5
NaK	275	6.2	513	6.9
NaSi	477	5.7	490	9.8
NaKSi	198	6.7	378	8.9
Cl × age means	322	6.5	439	9.1
LSD (Student) 1 % level	176	n.s.	171	n.s.
Variety × age × Cl means				
U.S. 22/3	340	6.6	462	9.1
GW 304	304	6.5	417	9.1
F-values **				
K	80.5	0.5	19.8	5.7
Si	0.0	0.0	1.1	0.4
Na	4.0	1.4	9.5	0.5
Variety	1.4	0.0	2.4	0.0

* K, Na, Si refer to the high level treatment.
** See table II for required F-values.

any significant variety-nutrient interactions, indicating that both varieties performed similarly at either Cl, Na, K, or Si level.

GROWTH AS A FUNCTION OF CHLORIDE OR BROMIDE SUPPLY: The results of the factorial experiment showed that Cl ions increased growth when added to modified Hoagland's No. 1 culture solution (table I) prepared from chemically pure salts. The growth increases were accompanied by much higher Cl concentrations within the plant than found in comparable plants without Cl added to the culture solutions. The exact amount required to overcome a Cl deficiency was investigated in the next experiment.

The plants were again grown in culture solutions to which increasing amounts of Cl ion, as KCl, were added to the basic culture solution. As before, a basic culture solution prepared from chemically pure salts and distilled water served as a control. Approximately 0.013 meq Cl as the Cl ion was present as an impurity in a total of 20 l of solution.

In addition to the Cl series of plants another set of cultures was prepared containing KBr at a concentration of 0.001 M (treatment 10, table IV). In treatment 11 the plants received both Cl and Br at 0.001 M concentrations. All plants were grown from June 8 to August 2, 1954. On August 2 the plants were harvested, and the results obtained for each Cl level and Br addition are given in table IV. The Cl concentrations of the petioles and blades of recently matured leaves and their relation to plant growth and sucrose content are given in figures 3, 4, 5, and 6.

The addition of Cl as Cl^- to the culture solutions again had a large effect upon the growth of the sugar beet plants (table IV). The tops increased from an average value of 402 gm per tank for the solutions containing only 0.013 meq of Cl per 20 l as an impurity (treatment 1) to 705, 771, and 776 gm for solutions that received 20, 60 and 180 meq Cl per 20 l, respectively (treatments 7, 8, and 9). The addition of Br as Br^- at a concentration of 20 meq per 20 l (treatment 10) resulted in a top growth equal to that of the comparable Cl^- treatment (treatment 7). With both Br and Cl present at 0.001 M concentrations, the top weights were again equal to treatments 7 and 10.

The dry weights of the tops are similar in pattern

TABLE IV

EFFECTS OF CHLORINE AND BROMINE ON GROWTH, SUCROSE CONCENTRATION OF STORAGE ROOTS AND ON THE PETIOLE AND BLADE CHLORINE OF SUGAR BEET PLANTS (MEANS OF 5 REPLICATIONS)

TREATMENT			TOPS		OLD LEAVES DRY	STORAGE ROOTS			FIBROUS ROOTS		CHLORINE	
No.	Cl ADDED	Br ADDED	FRESH	DRY		FRESH	SUCROSE		FRESH	DRY	MATURE PETIOLES	MATURE BLADES
	meq	*meq*	*gm*	*gm*	*gm*	*gm*	*gm*	*%*	*gm*	*gm*	*μeq/gm*	*μeq/gm*
1	0.00	0.0	402	48.4	4.62	269	26.4	9.8	48	4.00	4.9	3.3
2	0.08	0.0	489	59.1	4.09	291	30.5	10.4	54	4.58	6.4	5.0
3	0.25	0.0	522	61.6	5.26	359	38.6	10.8	66	5.50	8.1	4.6
4	0.74	0.0	523	61.8	4.32	376	40.9	10.9	55	4.82	12.4	5.4
5	2.22	0.0	603	68.0	4.11	379	42.3	11.2	67	5.71	23.6	8.9
6	6.67	0.0	636	72.3	4.82	446	51.1	11.4	79	6.49	54.0	13.9
7	20.00	0.0	705	73.6	4.62	464	48.1	10.4	82	6.45	185.7	29.7
8	60.00	0.0	771	81.5	5.93	423	44.7	10.5	99	7.76	617.9	95.4
9	180.00	0.0	776	76.1	4.38	517	52.0	10.0	90	6.79	1345.1	269.7
10	0.00	20.0	727	78.3	5.15	415	43.9	10.6	83	6.83	11.0	4.4
11	20.00	20.0	737	76.6	5.69	498	53.8	10.8	94	7.59	179.7	25.7
LSD (Student) 1 % level			97	9.2	n.s.	78	9.2	0.9	24	1.98	....	....

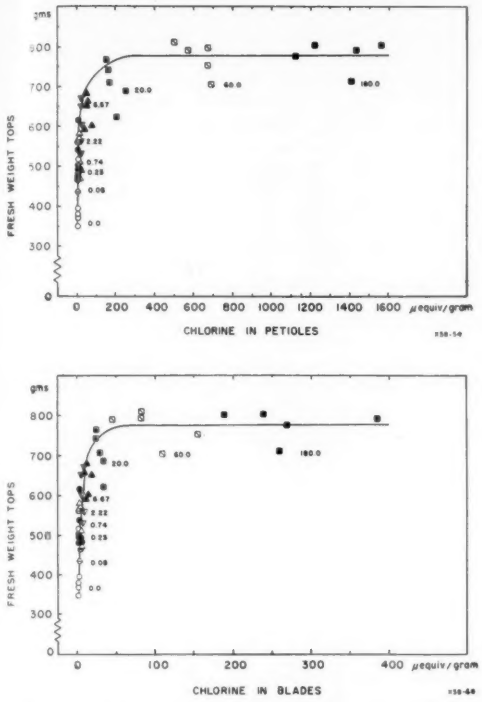

FIG. 3 (*above*). Relation of petiole Cl values of recently matured leaves (dry basis) to the fresh weight of the tops. The numbers in the graph refer to mg atoms of Cl added per 20 l of nutrient solution. The critical petiole Cl concentration, below which top growth decreases, is approximately 200 µeq/gm dry tissue.

FIG. 4 (*below*). Relation of Cl values for the upper upper half of the blades of recently matured leaves to the fresh wt of the tops. The critical blade Cl concentration, below which top growth decreases, is approximately 50 µeq/gm dry material. See also figure 3.

to the fresh weights. The dry weights of the old leaves did not differ significantly from each other.

The beet root weights, in contrast to the results of the factorial experiment, were nearly doubled by the higher Cl additions to the culture solutions. The addition of Br^- at a concentration of 0.001 M to the basic culture solution (treatment 10) resulted in a lower beet root weight than in the comparable Cl concentration (treatment 7) but the decrease was not significant statistically.

The fresh and dry weights of the fibrous roots followed a pattern similar to the storage root weights (table IV). Whereas the appearance of the storage roots was not altered by a Cl deficiency the fibrous roots of deficient plants had many stubby lateral branches in contrast to the filamentous character of roots from plants well supplied with chlorine (fig. 2). The substitution of Br for Cl resulted in fibrous roots that had the same appearance and weight as the Cl treated plants.

Particularly interesting was the observation that when Br^- was added to low Cl solutions there was a complete absence of Cl deficiency symptoms and the growth of tops and roots was excellent. Chloride contamination of the Br salts is hardly the explanation for this good growth, since there was none found in the KBr salts added to the culture solutions. The blade Cl value of 4.4 µeq/gm (treatment 10, table IV) is far below that found for non-deficient plants and the petiole Cl value of 11.0 µeq/gm would indicate a top yield of only 522 gm (treatment 4, table IV) instead of the 727 gm found for the tops of the Br treated plants (treatment 10). Similar comparisons can be made for storage and fibrous root growth. Apparently, these results indicate that Br^- can substitute for Cl^- in the growth of tops and roots.

The Cl values for the petioles and blades of plants in treatment 11 (Cl + Br) agree with those for treatment 7 which received Cl only. The addition of Br to Cl at this concentration did not influence Cl absorption.

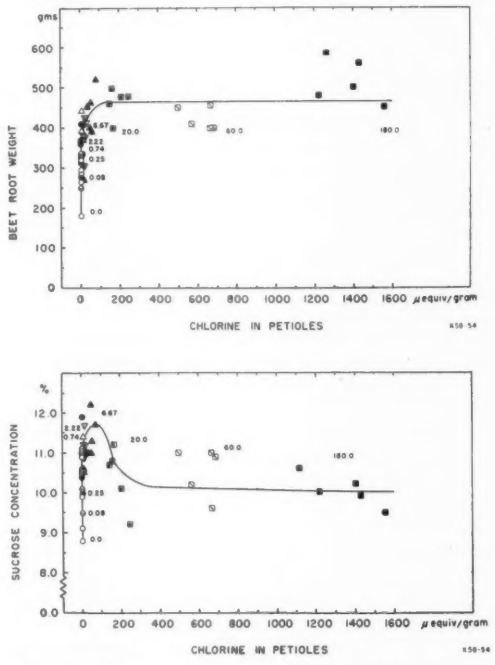

FIG. 5 (*above*). Relation of petiole Cl values of recently matured leaves (dry basis) to the fresh weight of storage roots. The critical petiole Cl concentration, below which storage root growth decreases, is approximately 200 µeq/gm dry material. See also figure 3.

FIG. 6 (*below*). Relation of petiole Cl values of recently matured leaves (dry basis) to the sucrose concentration of storage roots. Sucrose concentrations of the storage root are depressed by an extreme Cl deficiency, increased sharply by a mild deficiency, and lowered by an ample supply of Cl. See also figure 3.

GROWTH AS A FUNCTION OF PETIOLE AND BLADE CHLORIDE CONCENTRATION: The Cl concentrations in the petioles of recently matured leaves are given in table IV as μeq per gram of dry substance. A statistical analysis of the logarithms of the concentrations indicate that a difference of 0.14 expressed as a common log is necessary for significance at the 1 % level. On this basis the values for treatments 1 and 2, and 2 and 3 are alike, but there is a significant difference between 1 and 3. Significant increases in Cl concentrations took place between treatments 4 through 9. For the blades, the Cl values for the first 3 treatments did not differ significantly from each other. Each increment of Cl added thereafter produced a significant increase in Cl concentration in most instances (table IV).

The relation of petiole Cl to top growth is given in figure 3. Top growth begins to be retarded at petiole Cl concentrations of 100 to 200 μeq/gm (critical concentrations) and pronounced deficiencies are indicated by values of less than 10 μeq/gm. Plants with petioles containing 200 μeq/gm and above are adequately supplied with Cl (fig 3 and table IV). Similar conclusions about top growth and Cl concentrations can be drawn from the values for the leaf blades (fig 4 and table IV). The critical range is from 25 to 50 μeq/gm of dry blade tissue; approximately ¼ that of the petioles. For extreme Cl deficiencies the Cl concentrations of the blades are only slightly less than those of the petioles but at the higher Cl levels, the blade values are only 1/5 that of the petiole values. Thus the petioles are far more sensitive to Cl changes within the beet plant than the corresponding blades, and therefore petiole analysis appears preferable to blade analysis as a means of measuring the chlorine status of sugar beet plants.

The relation of petiole Cl to storage root growth is given in figure 5. The Cl values relative to storage root growth are quite similar to those for top growth. The critical Cl value is again approximately 200 μeq of Cl per gram of dry tissue, with the plants above this being well supplied with Cl and those with values of less than 10 μeq/gm indicating an extreme Cl deficiency.

PETIOLE AND BLADE BROMIDE CONCENTRATIONS: The petioles of the plants receiving Br only (treatment 10) contained 87.1 μeq of Br per gm of dry plant tissue (average of 5 replications) and the corresponding blades 11.0 μeq/gm. The Br values for the Cl plus Br treatment (treatment 11) are 157.9 μeq/gm for the petioles and 35.1 μeq/gm for the blades. As would be expected, the combined Cl and Br values correspond to those which lie between treatments 7 and 8 for Cl additions alone (table IV). However, still unexplained are the much higher Br concentrations for the petioles of the plants treated with Cl and Br (treatment 11) than for those treated with Br only (treatment 10). One explanation is that this is just a chance observation that is associated with the plant and another explanation is that it is associated with analytical variation but this is very unlikely because the values for duplicate samples agreed very closely with each other. While the yields for the Br treated plants are somewhat higher than what would be expected for a petiole halide concentration of 98.1 μeq/gm (between treatments 6 and 7, table IV) this may be due to chance and not to a real effect of Br.

TABLE V

NUTRIENT GRADIENTS WITHIN SUGAR BEET LEAVES EXPRESSED AS MICROGRAM ATOMS PER GRAM DRY TISSUE

PLANT PART	Cl	SO_4-S	NO_3-N	PO_4-P*	K	Na
Upper blade ...	78	151	46	77	396	1500
Lower blade ...	366	128	106	82	407	1710
Mid-rib of blade	694	63	436	54	417	1820
Upper petiole ..	938	21	715	72	524	1780
Middle petiole	1079	20	680	70	570	1750
Lower petiole ..	1262	24	609	72	650	1830
Entire blade ..	260	126	169	68	417	1610

* Phosphate-phosphorus soluble in 2 % acetic acid.

SUCROSE FORMATION RELATIVE TO CHLORIDE OR BROMIDE SUPPLY: As may be seen in table IV and figure 6, Cl deficient plants are significantly lower in sucrose concentration than comparable plants with an adequate supply of this element. Apparently, Cl is involved in the formation of sugar rather than in sucrose utilization. If Cl had been related to sugar utilization, then a deficiency of Cl would have caused an increase in sucrose concentration of the beet root, analogous to that of a N deficiency (34). Increasing the Cl supply still more, however, leads to a decrease in sucrose concentration of the storage root (treatments 7, 8, and 9). The cause of this decrease is not known.

Replacing Cl by Br (treatment 10, table IV) or by adding Br and Cl salts together at equal concentrations (treatment 11), caused no significant change in the sucrose concentration of the beet roots (treatments 7, 10 and 11). This indicates that Br may substitute for Cl not only in top and root growth but also in maintaining the sucrose concentration of the beet roots.

DISTRIBUTION OF CHLORIDE WITHIN THE PLANT: Within the tops of plants adequately supplied with Cl, the Cl concentrations of the blades differed little with respect to age of the leaf, being 51, 45 and 65 μeq/gm for young, mature and old leaves, respectively. In the petioles of the same leaves the Cl concentrations were very much higher than in the blades, being 279, 470 and 701 μeq/gm for the young, mature and old leaves, respectively. This increase in Cl concentration with the age of the petiole has been found to be typical of sugar beet plants studied thus far.

The chloride gradient within the leaves of field grown plants is indicated in table V for leaves separated into upper blades, lower blades, mid-ribs, and upper, middle, and lower sections of petioles. Within the petiole the Cl concentration decreases slowly from the base of the petiole to the mid-rib. The mid-rib itself, while appreciably lower in Cl than the petiole,

is much higher in Cl than the blade tissues. The upper blade is distinctly lower in Cl than the lower part of the blade. The reason for the sharp decrease in Cl concentration from the petiole to the blade tissues is not known, although the selective absorption by the petioles or the exclusion of Cl by the blade tissues could give rise to such concentration differences. A similar gradient for NO_3-N exists (table V), but here the decreases are quite likely associated with nitrate reduction and not with absorption barriers as seems to be indicated for the movement of Cl from conducting to blade tissues.

The marked decrease in Cl concentration from petioles to blades is apparently unique among the mineral constituents of sugar beet leaves (table V). Phosphate-phosphorus tends to be higher in the blades than in the petioles, especially when there is a P deficiency and when the plants are deficient in N (34). Similarly, when plants are deficient in K and contain ample quantities of Na, K concentrations of the blades are higher than in the petioles, but when the plants are deficient in other nutrients, the petioles frequently increase in K concentration far more rapidly than the blades. Sodium concentrations in these plants differ little from petioles to blades. In contrast to the Cl concentrations, SO_4-S concentrations are far higher in the blades than in the petioles.

CHLORIDE RECOVERY: The addition and recovery of Cl ions from sugar beet plants was studied in an experiment that involved only 2 treatments. In one treatment sugar beet plants were grown in modified Hoagland's culture solution which contained all elements known to be necessary to plant growth, including Na and Si but not Cl. The plants in the other treatment received the same nutrients, except for 20 meq of $SO_4^=$ from Na_2SO_4, which was replaced by 19.7 meq of Cl^- from NaCl. Sugar beet plants of the U.S. 22/3 variety at the early 2-leaf stage were set into the culture solutions on February 5, 1954. On April 22, 1954 the plants were separated into tops and roots. These parts were dried at 80° C and analyzed for Cl in triplicate by the modified Conway procedure.

The Cl recovered from the plants in each treatment is given in table VI. The tops and roots of the plants low in Cl contained 0.380 meq of Cl. The original culture solution contained 0.040 meq and the seedlings at the time of transplanting 0.009 meq or a total of only 0.049 meq of Cl. At the 1 % level, the lower confidence limit for Cl recovery in the plant is 0.250 meq of Cl. This value exceeds by far the Cl originally present (0.049 meq) as a contaminant. This implies that the plants accumulated Cl during the growth period from the distilled water added from time to time to the culture solutions or from other sources such as the air surrounding the plants. Since the distilled water supplied at most only 0.005 meq of Cl, the rest must have originated elsewhere.

The Cl recovered from the plants receiving 19.7 meq of Cl averages 19.8 meq (table VI). After subtracting 0.4 meq of Cl recovered from the minus Cl plants, a net recovery of 19.4 meq of Cl is obtained, which compares favorably to the amount added initially, 19.7 meq. The confidence limits for the net recovery of Cl from 6 sets of plants are 19.3 and 20.3 meq at the 5 % level and 19.0 and 20.7 meq at the 1 % level. This implies that there is a probability of 95 and 99 %, respectively, that these limits contain the true mean of Cl recovery for these plants. Since these limits cover the amount of Cl added initially, one may conclude that there has not been an appreciable absorption of extraneous Cl or a loss of Cl from the plant system, unless the losses and gains cancelled each other or occurred within the confidence limits of the present experiment.

CHLORINE DEFICIENCY SYMPTOMS ON PLANTS GROWN IN HOAGLAND'S NUTRIENT SOLUTION: The ease with which Cl deficiency symptoms can be induced in sugar beet plants, using technical grade chemicals, regular distilled water, and vermiculite as a substrate came as a surprise when demonstrating the growing of sugar beet plants by the "open culture" technique. In the demonstration, sugar beet seeds were planted in vermiculite at a depth of ¾ inches in November 1953 and thereafter watered daily with approximately one liter of half strength Hoagland's nutrient solution No. 1 (11). The excess solution drained off freely and was not recovered for later use. When the plants were 6 to 8 weeks old, symptoms similar to those illustrated in figure 1 developed on the blades of the young leaves. On February 24, 1954 the pots were divided into 2 lots. One lot was watered thereafter with half strength Hoagland's solution to which $CaCl_2$ at a concentration of only 0.1 meq of Cl per liter had been added, the other lot watered as before without the $CaCl_2$ addition. The deficiency symptoms were completely removed within

TABLE VI

CHLORINE RECOVERY FROM SUGAR BEET PLANTS

Pot	Tops dry wt gm	Roots dry wt gm	Tops Cl μEQ/GM	Roots Cl μEQ/GM	Tops Cl MEQ	Roots Cl MEQ	Total Cl MEQ
High chlorine plants (19.7 meq Cl^- added)							
69	107.9	101.6	179.2	7.88	19.34	0.80	20.14
72	118.5	67.8	160.3	11.66	19.00	0.79	19.79
85	109.0	115.0	169.7	10.00	18.50	1.15	19.65
102	105.9	69.9	175.0	9.55	18.53	0.67	19.20
119	112.5	138.7	163.7	12.99	18.42	1.80	20.22
Mean	110.8	98.6	169.6	10.42	18.76	1.04	19.80 *
Low chlorine plants (0.049 meq Cl^- impurity)							
80	62.6	62.5	3.95	1.90	0.25	0.12	0.37
88	91.1	75.0	3.58	1.04	0.33	0.08	0.41
93	62.3	53.4	4.23	0.94	0.26	0.05	0.31
106	89.1	93.8	3.89	1.22	0.35	0.11	0.46
113	65.4	70.3	3.85	1.19	0.25	0.08	0.33
Mean	74.1	71.0	3.90	1.26	0.29	0.09	0.38 **

* Confidence limits at the 5 % level, 19.29 to 20.31.
** Confidence limits at the 5 % level, 0.30 to 0.46

25 days after the addition of Cl^-. Those plants without the Cl added continued to retain and develop leaves with deficiency symptoms.

In the culture of sugar beets, it is recommended that Cl at a concentration of 5.0 meq per liter be added to Hoagland's solution either as $CaCl_2$ or as NaCl. The addition of NaCl is preferable to $CaCl_2$ whenever "normal" nutrition in relation to potassium and sodium is desired (unpublished results of this laboratory).

Discussion

Failure to observe chlorine deficiency symptoms on sugar beet plants earlier was undoubtedly due to the presence of Cl as a contaminant in the salts used to prepare the nutrient solutions and perhaps to the use of unfiltered air in contrast to greenhouse air now filtered through activated carbon. Still another factor is the similarity of the incipent Cl deficiency symptoms to those of manganese deficiency.

In the factorial experiment, involving Cl, Na, Si, K and variety of sugar beet, the first symptoms, which later proved to be associated with Cl, were visible only through transmitted light. These symptoms appeared on young and recently matured leaves and were similar to those of Mn deficiency. Even after adding 10 mg of Mn as $MnSO_4$ to each tank of culture solution, the deficiency symptoms persisted. A more careful inspection of the plants revealed that the symptoms appeared only on those plants to which no Cl had been added to the culture solution. As these plants became older symptoms could be seen readily on the high K-low Cl plants, and somewhat less readily on the center leaves of the low K-low Cl plants. If the K salts had contained more Cl as an impurity, the Cl deficiency symptoms would not have been observed in the low K plants nor even in the high K plants, since by adding K as K_2SO_4, Cl as a contaminant would have been added at the same time. One may assume that as the manufacturers of reagent and technical grade salts have improved their processes, salts lower in Cl have been produced, and now Cl must be added to nutrient solutions to prevent Cl deficiency of sugar beet plants.

Summary

Sugar beet plants were grown in nutrient solution prepared from low halide salts of the reagent grade. The first deficiency symptoms developed on the blades of the center leaves of the plant and in the early stages resembled those of Mn deficiency. Through transmitted light the symptoms appeared as a netted mosaic pattern, with the green veins forming the netting. As the symptoms developed, the interveinal areas appeared as smooth flat depressions, light green to yellow in color, and presented a striking contrast to the green veins which had a "raised" appearance. A feature of the Cl deficiency was the stubby growth of the secondary roots. Both the leaf and root symptoms were absent in culture solutions having the additions of Cl in the amounts of 6.7 to 20 meq per three plants and Br at the one amount used, 20 meq per three plants. The addition of Mn, Na, Si, as well as those salts contained in Hoagland's solution No. 1 failed to correct the symptoms.

Petiole Cl values of 4.9 to 7.9 μeq and blade Cl values of 3.3 to 5.4 μeq per gram of dry tissue were indicative of extreme Cl deficiency. Petiole Cl values of 200 μeq or above and blade (without mid-rib) Cl values of 50 μeq or above per gram of dry tissue were indicative of Cl adequacy.

Chlorine was necessary for top and root growth and was associated with sugar formation rather than with sugar utilization. The storage roots of plants deficient simultaneously in Cl and K were higher in sucrose concentration than non-deficient plants, whereas a deficiency of either nutrient alone resulted in a decrease in sucrose concentration. Still more Cl, however, depressed sucrose concentrations of the storage root but did not decrease root size.

Petiole Cl concentrations, but not those of the blades, increased with the age of the leaf. For plants well supplied with Cl, the Cl concentrations within the upper blade tissues of the leaf were less than one-tenth those of the corresponding petioles. Comparable decreases for NO_3-N were observed in the leaf blades, but this decrease was due to nitrate reduction, whereas the Cl decreases were thought to be associated with absorption barriers or to factors not now known. Sulfate, in contrast to Cl and NO_3, accumulated in blade tissues at far higher concentrations than in the corresponding petioles.

The Cl recovered from untreated plants was in excess of the Cl present as an impurity in the nutrient solutions and that contained in the transplanted seedlings, whereas complete recovery of chloride was indicated for plants treated with 20 meq of Cl after correcting for the Cl found in the untreated plants.

Chlorine deficiency symptoms were induced in sugar beet plants that were grown in the greenhouse by the "open culture" technique, using nutrient solutions prepared from chemically pure salts without special purification, regular distilled water, and vermiculite as a substrate.

The addition of Na to low K plants increased growth significantly but had no effect upon high K plants. The effects of silicon were not conclusive.

Literature Cited

1. Browne, C. A. and Zerban, F. W. Physical and Chemical Methods of Sugar Analysis. Pp. 359–373. John Wiley and Sons, Inc., New York 1941.
2. Broyer, T. C., Carlton, A. B., Johnson, C. M. and Stout, P. R. Chlorine—a micronutrient element for higher plants. Plant Physiol. 29: 526–532. 1954.
3. Conway, E. J. Microdiffusion Analysis and Volumetric Error. Pp. 182–205. D. Van Nostrand Company, Inc., New York 1950.
4. Cooper, H. P., Paden, W. R. and Phillippe, M. M. Effects of applications of sodium fertilizer on yields and composition of the cotton plant. Soil Sci. 76: 19–28. 1953.
5. Cope, J. T., Jr., Bradfield, R. and Peech, M. Effect

of sodium fertilization on yield and cation content of some field crops. Soil Sci. 76: 65–74. 1953.
6. CROWTHER, E. M. The effects of potassium and sodium fertilizers on the yield and composition of sugar beets. Mimeographed report, Chemistry Department, Rothamsted Experimental Station, England c. 1950.
7. EATON, F. M. Toxicity and accumulation of chloride and sulfate salts in plants. Jour. Agr. Research 64: 357–399. 1942.
8. GAMMON, N., JR. Sodium and potassium requirements of pangola and other pasture grasses. Soil Sci. 76: 81–90. 1953.
9. HARMER, P. M. and BENNE, E. J. Effects of applying common salt to a muck soil on the yield and quality of certain vegetable crops and on the composition of the soil producing them. Jour. Amer. Soc. Agron. 33: 952–979. 1941.
10. HARMER, P. M., BENNE, E. J., LAUGHLIN, W. M. and KEY, C. Factors affecting crop response to sodium applied as common salt on Michigan muck soil. Soil Sci. 76: 1–17. 1953.
11. HOAGLAND, D. R. and ARNON, D. I. The water-culture method for growing plants without soil. Agr. Expt. Sta., California, Circ. 347. 1950.
12. HOLT, M. E. and VOLK, N. J. Sodium as a plant nutrient and substitute for potassium. Jour. Amer. Soc. Agron. 37: 821–827. 1945.
13. JACOBSON, L. Maintenance of iron supply in nutrient solutions by a single addition of ferric potassium ethylenediamine tetra-acetate. Plant Physiol. 26: 411–413. 1951.
14. JOHNSON, C. M. and NISHITA, H. Microestimation of sulfur in plant materials, soils, and irrigation waters. Anal. Chem. 24: 736–742. 1952.
15. JOHNSON, C. M. and ULRICH, A. Determination of nitrate in plant material. Anal. Chem. 22: 1526–1529. 1950.
16. KRETSCHMER, A. E., TOTH, S. J. and BEAR, F. E. Effect of chloride versus sulfate ions on nutrient-ion absorption by plants. Soil Sci. 76: 193–199. 1953.
17. LANCASTER, J. D., ANDREWS, W. B. and JONES, U. S. Influence of sodium on yield and quality of cotton lint and seed. Soil Sci. 76: 29–40. 1953.
18. LARSON, W. E. and PIERRE, W. H. Interaction of sodium and potassium on yield and cation composition of selected crops. Soil Sci. 76: 51–64. 1953.
19. LEHR, J. J. Importance of sodium for plant nutrition I. Soil Sci. 52: 237–244. 1941.
20. LILL, J. G., BYALL, S. and HURST, L. A. The effect of applications of common salt upon the yield and quality of sugar beets and upon the composition of the ash. Jour. Amer. Soc. Agron. 30: 97–106. 1938.
21. LIPMAN, C. B. Importance of silicon, aluminum, and chlorine for higher plants. Soil Sci. 45: 189–198. 1938.
22. LUNT, O. R. and NELSON, W. L. Studies on the value of sodium in the mineral nutrition of cotton. Soil Sci. Soc. Amer., Proc. 15: 195–200. 1950.
23. MARSHALL, J. G. and STURGIS, M. B. Effects of sodium fertilizers on yield of cotton. Soil Sci. 76: 75–79. 1953.
24. MULLISON, W. R. and MULLISON, E. Growth responses of barley seedling in relation to potassium and sodium nutrition. Plant Physiol. 17: 632–644. 1942.
25. PIPER, C. S. Soil and Plant Analysis. Pp. 268–269. Interscience Publishers, Inc., New York 1944.
26. RALEIGH, G. J. Evidence for the essentiality of silicon for growth of the beet plant. Plant Physiol. 14: 823–828. 1939.
27. RALEIGH, G. J. Silicon in plant growth. Soil Sci. 60: 133–135. 1945.
28. RALEIGH, G. J. Effects of the sodium and of the chloride ion in the nutrition of the table beet in culture solutions. Proc. Amer. Soc. Hort. Sci. 51: 433–436. 1948.
29. RUSSELL, E. J. Soil Conditions and Plant Growth, 8th ed. Chapt. III. Longmans, Green and Company, New York 1950.
30. SNEDECOR, G. W. Statistical Methods Applied to Experiments in Agriculture and Biology. Pp. 1–485. The Collegiate Press, Ames, Iowa 1946.
31. TRUOG, E., BERGER, K. C. and ATTOE, O. J. Response of nine economic plants to fertilization with sodium. Soil Sci. 76: 41–50. 1953.
32. TULLIN, V. Response of the sugar beet to common salt. Physiol. Plantarum 7: 810–834. 1954.
33. ULRICH, A. Chap. 6. In: Diagnostic Techniques for Soils and Crops, H. B. Kitchen, ed. Pp. 157–198. American Potash Institute, Washington 6, D. C. 1948.
34. ULRICH, A. Influence of night temperature and nitrogen nutrition on the growth, sucrose accumulation and leaf minerals of sugar beet plants. Plant Physiol. 30: 250–257. 1955.
35. WALLACE, A., TOTH, S. J. and BEAR, F. E. Influence of sodium on growth and composition of Ranger alfalfa. Soil Sci. 65: 477–486. 1948.
36. WEHUNT, R. L. and COLLINS, W. O. Response of oats to Na and K on Norfolk sandy loam at two residual K levels. Soil Sci. 76: 91–96. 1951.

THE LARGE SCALE LABORATORY CULTURE OF CHLORELLA UNDER CONDITIONS OF MICRONUTRIENT ELEMENT DEFICIENCY [1]

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Elucidating the metabolic roles of the micronutrient elements through analysis of both normal and deficient tissues for products of metabolism and for enzymes may provide clues as to the function of the various elements.

The unicellular alga, Chlorella, was chosen as a test organism for this purpose because it can be grown readily under controlled concentrations of micronutrients and can be sampled quite accurately. A survey of the literature revealed no extant Chlorella culture apparatus which was easily adaptable for the

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above-stated purpose. Sterile, intermittent sampling from large flasks, such as used by Mandels (7), is difficult because aliquots must be withdrawn from the top. The culture vessels used by Hopkins (4, 5), Hopkins and Wann (6) and Walker (13) do not provide large quantities of material. The constant culture apparatus of Myers and Clark (10) is too complicated for use in experiments requiring a number of simultaneous treatments. In the monograph of Burlew (1) are included several devices for growing Chlorella on a large or small scale. The small scale devices do not produce enough total Chlorella or are highly specialized with respect to one aspect of Chlorella growth (2, 12). The large scale systems have inadequate controls for aseptic micronutrient work (8).

The problem of producing sufficient quantities of cellular material for analysis was solved by the development of a Chlorella culture unit which would:

(a) Produce large quantities (about 20 gm fresh weight) of material in a short time,

(b) Provide a means of intermittent sampling under sterile conditions,

(c) Maintain continuously a uniform suspension of cells free from living contaminants, and

(d) Permit the demonstration of growth under micronutrient deficient conditions.

Several of these culture units have been incorporated into a system in which uniform growth rates have been obtained among several cultures. Furthermore, iron and manganese deficiencies have been demonstrated.

Materials and Methods

The complete system consists of 6 culture units maintained under conditions uniform with respect to CO_2 supply, light intensity, and temperature.

The culture unit, shown in detail in figure 1, consists of a large Pyrex cylinder with a three-way stopcock at the bottom connected to an inlet filter and with a 3-way outlet piece at the top. To prevent loss of liquid through the outlet piece (due to foaming in the later stages of growth), the liquid capacity of the units is held to 2 liters. Air containing 2 % CO_2 is passed through the culture unit, as indicated in figure 1, in order to maintain a homogeneous cell suspension and to allow a high rate of growth.

A 60-cm (24-inch) long inlet filter is necessary to exclude particulate contaminants during the extended periods of aeration. A plug of acid-washed glass wool at the base of the cotton prevents loose cotton fibers from entering the suspension.

The glass bulb below the filter provides a safety reservoir which prevents the wetting of the filter packing by backflow of liquid during periods of low air pressure at the onset of aeration.

The inlet tube is connected to one tube on the double side of the stopcock and a capillary connects the single side of the stopcock and the base of the growth chamber. The thick-walled capillary tube serves to maintain uniform bubble size among all the

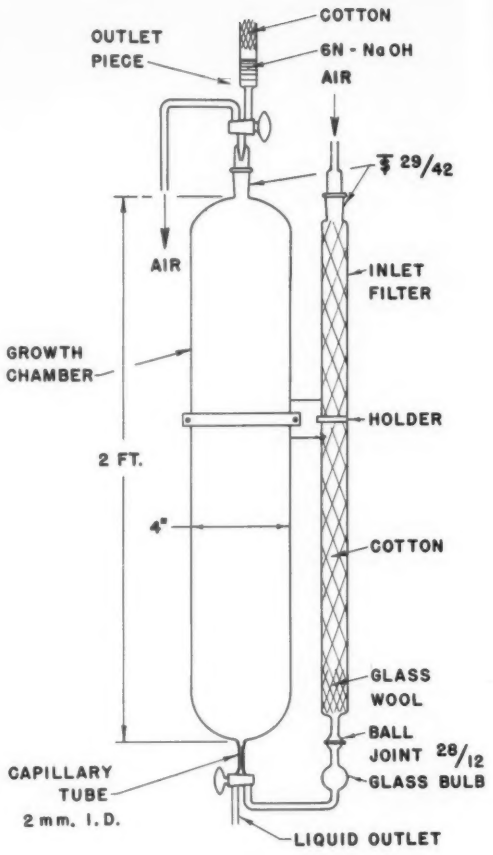

Fig. 1. Chlorella culture unit.

cultures. This construction also reduces the contact of the solution with the stopcock grease which might introduce contaminants. The other tube of the double side of the stopcock is open and is utilized for sampling. The stopcock controls alternately the incurrent air and the excurrent suspension. The downward flow of the suspension permits easy withdrawal of aliquots with minimum danger of foreign organisms entering the chamber.

During normal operation, air flows freely through the open tube of the outlet piece. During sampling, the outlet piece stopcock is closed, and internal air pressure forces the liquid out rapidly through the liquid outlet tube. In addition, the cotton-plugged reservoir of the outlet piece contains a sterile solution of 6 *N* NaOH. This reserve of base is used to adjust the pH of cultures provided with ammonia nitrogen during the growth period.

Aeration of the suspension is accomplished by the system illustrated in figure 2.

The combination of the oil-and-water trap and the long, glass-enclosed filter serves to cleanse the air of the major portion of its particulate matter content.

The use of two pressure reducing valves in series with the air compressor, the large buffer tanks, and the mercury pressure relief valve reduces the rather large fluctuations in pressure characteristic of the tank-type compressor. Air flow from the manifold to each culture unit is controlled by a glass stopcock so that the maximum difference in flow is about 10 %. Closer regulation is obviated by the enrichment of the air with CO_2 which keeps the supply of this compound from being a limiting factor in the growth of the organisms. The CO_2 is supplied from a cylinder at 2 % of the total gas flow by regulation with a needle valve and continuous metering with a sensitive orifice flow meter. The flow rate is measured from the outlet piece by means of an orifice type flow meter. Air supplied at the rate of 100 ml/minute per culture unit has been found adequate for rapid growth and the maintenance of a homogeneous cell suspension.

Light is provided by four 8-ft fluorescent lamps placed about 22.5 cm (9 inches) away from the units. The culture units are distributed evenly in front of the lamps so that the end units are at least 45 cm (18 inches) in from the ends of the lamps. The luminous intensity at the units is about 400 fc.

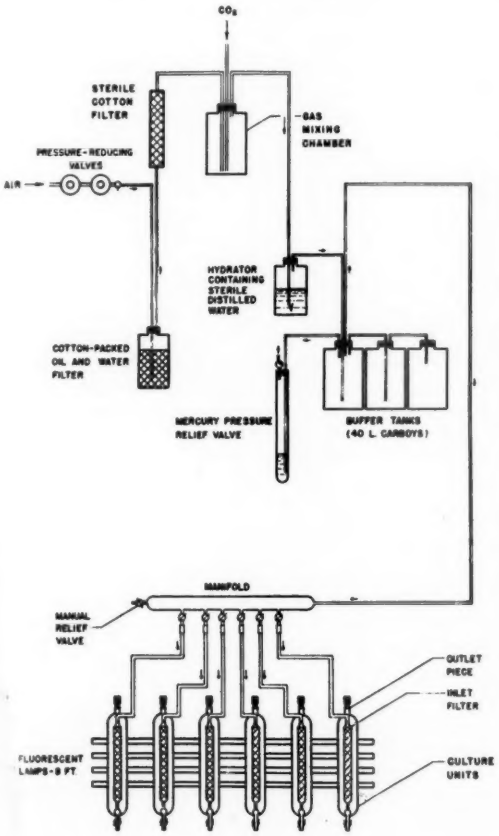

FIG. 2. Chlorella culture system.

The entire system is placed in a temperature controlled room maintained at 21° C. Because of absorption of radiant energy and local heating by the fluorescent lamp circuit, the temperature of the suspensions is about 24° C.

All glassware is cleaned by steaming with a 1 : 1 aqueous solution of HNO_3 followed by repeated rinsings with water deionized by passage through a mixed bed of ion-exchange resins. The culture units are heated at 100° C for 4 hours in a dry heat soil drying oven. At the end of this time the solutions, which have been autoclaved at 15 psi for one hour, are poured, while still hot, into the culture units. The filled chambers are replaced into the drying oven and maintained at 100° C for an additional hour. Immediately before and after withdrawing aliquots from each culture unit the liquid outlet tube of the 3-way stopcock is rinsed out with 80 % ethanol from a plastic wash bottle.

Chlorella vulgaris var. *viridis* (Type culture collection, Cambridge University, Cambridge, England) is maintained on dextrose agar slants. Inoculum cultures are grown in a full nutrient solution (15). The inoculum is prepared from these cultures by centrifuging the cells, decanting the nutrient solution, and resuspending the cells in distilled water under aseptic conditions. The solutions in the culture units are inoculated by sterile pipette.

For early stages of growth, either of two methods of growth measurement is used. If there is considerable background turbidity in the solution, cell counts are taken by means of a haemocytometer chamber. If the solutions are free of background turbidity, measurements of turbidity are taken by means of a colorimeter devised by Ellis and Brandt (3) using 5 cm cuvettes and Corning filters #3385, #4303, and #5030 (520 mμ). These turbidity readings are referred to a standard curve derived from a series of dilutions of a suspension of known packed cell volume as determined by thrombocytocrit measurements (9). In the later stages of growth, packed cell volume is determined in the same manner, using an Evelyn colorimeter with 1.5 cm light path at 515 mμ. At various intervals during a run turbidimetric measurements are checked against direct thrombocytocrit measurements.

An iron deficient medium was prepared using macronutrient compounds purified by the phosphate adsorption technique of Stout and Arnon (11). The micronutrient compounds were used without special purification.

A manganese deficient medium was prepared as above for the iron deficiency test, with the exception of $MgSO_4$, which was obtained by treating sublimed magnesium (Dow Chemical Company) with distilled sulphuric acid.

RESULTS

TEST OF UNIFORMITY AMONG CULTURE UNITS: In order to test the uniformity of growth among the culture units, Chlorella was grown using the nutrient

solution of Winokur (15), having the following composition: 0.025 M KNO_3, 0.018 M K_2HPO_4, 0.020 M $MgSO_4 \cdot 7\,H_2O$, 0.44 ppm Mn, 0.08 ppm Zn, 0.002 ppm Cu, 0.02 ppm Mo, 7×10^{-6} M Fe citrate, pH 5.6. This basal nutrient solution was used in all experiments except for the omission of specific micronutrients.

Two liters of solution were put into each culture unit. Each unit was inoculated from a single liquid-medium stock culture so that the initial population was equivalent to 0.004 mm^3 cells per ml. The cultures in the culture units were grown for 13 days and growth measured turbidimetrically as described above.

As seen in table I, the maximum coefficient of variation among the chambers during the entire growth period was 13 %, with a minimum coefficient of variation of 6.1 %. The growth of Chlorella was quite rapid, rising from 0.004 to about 6 mm^3/ml in 13 days.

Iron and Manganese Tests: It has been possible to attain marked deficiencies of iron and manganese in these culture units.

Growth of cultures with and without added iron is shown in figure 3. At the end of the experiment, the control cultures had about 50 times the amount of Chlorella, on the basis of packed cell volume, as the iron-deficient cultures. The curves also indicate that there was no increase in iron in the deficient cultures during the experiment. However, the fact that there was some growth showed that iron was not completely eliminated from the nutrient solution.

The growth on the manganese-deficient medium is presented in figure 3, where it is shown that, in spite of the very low requirement of Chlorella for this element (14), essentially no growth was obtained over an extended time period. This shows not only the initial freedom of the deficient cultures from manganese, but the absence of cumulative manganese contamination during 8 days of vigorous aeration.

After 196 hours had elapsed, and the control and deficient cultures appeared to have attained a static growth rate, 8 μgm Mn/l solution was added to the deficient culture. As is seen in the curve, after a 3-day induction period, there was a sharp rise in the rate of growth of this culture followed by a levelling off at a population approximately one-half that of the control culture.

From the population density of the culture after the addition of manganese it is seen that the manganese level was still below the optimum.

Eight μgm Mn/l solution induced the growth of 5.7 ml of packed cells per liter. Since the dry weight was determined to be 490 mg/ml packed cell volume, the manganese requirement is 2.9 μgm/gm dry weight. This is in good agreement with the value obtained by Walker (14).

Maintenance of Sterility: Samples of the suspensions were tested for contamination at the end of the experiment by inoculation into a brom cresol purple base broth containing 1 % glucose and incubation at 37° C for 48 to 72 hours, with periodic microscopic examination of the media for bacterial growth. In none of the experiments described above was any indication of contamination by a foreign organism noted.

In a preliminary test of the apparatus, a sterile tryptose broth was placed in a culture unit which had been sterilized as above. The uninoculated broth was then aerated for two weeks. The nutrient medium remained clear during the entire run, and no evidence of microorganisms was found on staining and microscopic examination of the medium.

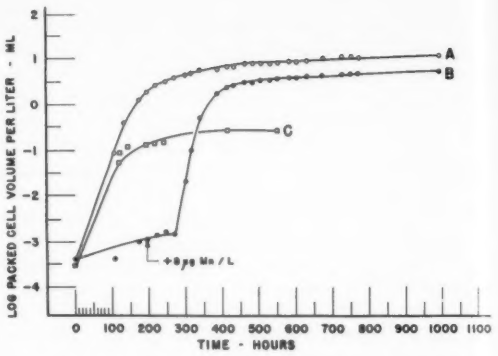

Fig. 3. Growth of Chlorella under normal and micronutrient element deficiency conditions.—A. Growth under full nutrient conditions.—B. Growth under manganese deficiency.—C. Growth under iron deficiency.

Table I

Growth of Six Cultures of Chlorella vulgaris viridis under Uniform Conditions of Light, Temperature and Nutrition

Time	Mean packed cell volume	Coeff of variation
hrs	*mm^3/ml*	*%*
0	0.004	0.0
46	0.019	7.4
70	0.066	6.1
99	0.63	13
199	1.3	10
142	2.1	11
166	2.8	9.3
191	3.4	6.2
214	4.3	7.7
238	4.4	11
284	5.5	7.3
310	6.0	8.3

Discussion

The apparatus described in this paper was designed to give the physiologist a means of studying, in a single experiment, a number of discrete effects of micronutrient element deficiency on the metabolism of Chlorella over an extended time period.

Because of the uniformity of growth performance among the units, only a small number of replications

is needed for each treatment. Also, the treatments can be modified during the course of the experiment without loss of sterility. Sampling can be accomplished safely with great rapidity to study the effects of treatment during any time interval. Finally, the large quantities of material available from individual replications allow the analysis of the cells for metabolic products as well as the testing of the cells for specific enzyme activities.

Summary

An apparatus has been devised for the growth of Chlorella which incorporated the following qualities:

1. It can provide large quantities of cellular material.
2. It allows the growth of Chlorella at a high enough rate to demonstrate growth differences among different treatments within a two-week period.
3. It allows the growth of Chlorella under conditions of micronutrient element deficiencies.
4. It provides a means of sterile, intermittent sampling of a single culture.
5. It allows the sterile addition of materials to a culture during an experiment.

The sublimed magnesium was donated by the Dow Chemical Company, Magnesium Department. This gift is gratefully acknowledged.

Literature Cited

1. Burlew, John S. Algal Culture: From Laboratory to Pilot Plant. Carnegie Inst. Wash. Publ. No. 600. 1952.
2. Davis, E. A., Dedrick, J., French, C. S., Milner, H. W., Myers, Jack, Smith, J. H. C. and Spoehr, H. A. Laboratory experiments on Chlorella culture at the Carnegie Institution of Washington, Department of Plant Biology. In: Algal Culture: From Laboratory to Pilot Plant, J. S. Burlew, ed. Carnegie Inst. Wash. Publ. No. 600: 105–153. 1952.
3. Ellis, Gordon H. and Brandt, C. S. Photoelectric colorimeter for use in microanalysis. Anal. Chem. 21: 1546–1548. 1949.
4. Hopkins, E. F. Iron-ion concentration in relation to growth and other biological processes. Bot. Gaz. 89: 209–240. 1930.
5. Hopkins, E. F. The necessity and function of manganese in the growth of Chlorella. Science 72: 609–610. 1939.
6. Hopkins, E. F. and Wann, F. B. Iron requirements for Chlorella. Bot. Gaz. 81: 407–427. 1927.
7. Mandels, Gabriel A quantitative study of chlorosis in Chlorella under conditions of sulphur deficiency. Plant Physiol. 18: 449–462. 1943.
8. Mituya, A., Nyunoya, T. and Tamiya, H. Pre-pilot-plant experiments on algal mass culture. In: Algal Culture: From Laboratory to Pilot Plant, J. S. Burlew, ed. Carnegie Inst. Wash. Publ. No. 600: 273–281. 1952.
9. Myers, Jack Culture conditions and the development of the photosynthetic mechanism. III. Influence of light intensity on the cellular characteristics of Chlorella. Jour. Gen. Physiol. 29: 419–427. 1946.
10. Myers, Jack and Clark, L. B. Culture conditions and the development of the photosynthetic mechanism. II. An apparatus for the continuous culture of Chlorella. Jour. Gen. Physiol. 28: 103–112. 1944.
11. Stout, P. and Arnon, D. I. Experimental methods for the study of the role of copper, manganese, and zinc in the nutrition of higher plants. Amer. Jour. Bot. 26: 144–149. 1939.
12. Tamiya, H., Hase, E., Shibata, K., Mituya, A., Iwamura, T., Nihei, T. and Sasa, T. Kinetics of growth of Chlorella, with special reference to its dependence on quantity of available light and on temperature. In: Algal Culture: From Laboratory to Pilot Plant, J. S. Burlew, ed. Carnegie Inst. Wash Publ. No. 600: 204–232. 1952.
13. Walker, J. B. Inorganic micronutrient requirements of Chlorella. I. Requirements for calcium (or strontium), copper and molybdenum. Arch. Biochem. Biophys. 46: 1–11. 1953.
14. Walker, J. B. Inorganic micronutrient requirements of Chlorella. II. Quantitative requirements for iron, manganese and zinc. Arch. Biochem. Biophys. 53: 1–8. 1954.
15. Winokur, M. Growth relationships of Chlorella species. Amer. Jour. Bot. 35: 118–129. 1948.

FLORAL INITIATION IN CESTRUM NOCTURNUM. I. A LONG-SHORT DAY PLANT[1]

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Since the first investigation of Garner and Allard (6) we have known of LDP[3] and SDP. Recently Resende (13) reported that floral initiation in several plant species is promoted by a short period of LD followed by SD and called this group the LSDP. In experiments with *Cestrum nocturnum* it was found that this species, too, is a LSDP. The purpose of this paper is to present an analysis of its photoperiodic requirements and to discuss the bearings of the results on the problems of synthesis of the floral stimulus and of intermediate day plants.

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[3] Abbreviations used: LDP, long day plant(s); SDP, short day plant(s); LSDP, long-short day plant(s); SLDP, short-long day plant(s); LD, long day(s); SD, short day(s); PP, photoperiod(s); NP, nyctoperiod(s).

Materials and Methods

All experiments were performed in the Earhart Laboratory making use of the temperature controlled greenhouses and artificial light rooms (22). The light intensity was generally in excess of 1500 fc in the greenhouses and approximately 700 fc in the artificial light rooms (all light intensities refer to that at the plant tops). Artificial light was supplied by warm white fluorescent tubes supplemented with incandescent bulbs. Unless otherwise noted LD refers to a 16-hour PP (8 hrs in the 26° C greenhouse plus 8 hrs of artificial light and an 8-hr NP at 20° C) and SD refers to an 8-hour PP (8 hrs in the 26° C greenhouse plus a 16-hr NP at 20° C).

Cestrum nocturnum, henceforward referred to by its generic name alone, is a member of the Solanaceae. It is a woody perennial shrub indigenous to the Central American tropics; Schulz (16) has reported its occurrence in the wooded mountainous areas of Cuba, Jamaica, and Martinique. It is widely grown in Southern California for ornamental purposes and is commonly known as the night-blooming jasmine.

All Cestrum plants used in these experiments were raised from seeds harvested in the vicinity of Pasadena. After 3 months growth in standard SD conditions the seedlings were ready for experimentation. At the end of each experiment the plants were cut back and the new shoots arising from the crown were cultivated; in this manner the same plants were used in as many as 4 separate experiments with no apparent carryover of the effects of previous inductive treatments. The plants were grown with a single main axis by continuous pruning of the elongating axillary branches. Generally each seedling had developed more than 35 nodes and attained a height in excess of 75 cm at the experimental age. The selection of plants was based on the size and number of leaves at the start of LD induction; at least 3 leaves greater than 110 mm long and, counting basipetally, 14 to 16 leaves total were required before a plant was used. The leaves counted were those visible without dissection of the apex and all leaves below the 14th to 16th were removed prior to the beginning of LD induction.

Three criteria were employed to evaluate quantitative differences in the flowering responses of plants; the percent of plants flowering (% Fl); the percent of plants with terminal flowers (% Term Fl), and the number of nodes flowering per plant (Nodes Fl/Pl). The latter method was first applied by Hamner in his studies with soybean (5) and reflects the amount of floral stimulus available for translocation from the leaves to the growing points (the errors quoted in the tables for this figure are the standard errors of the means). It has been shown that the terminal 5 leaves (less than 10 mm long) do not produce the floral stimulus (15) and, consequently, flowering at the terminal apex is always a result of translocation of the stimulus from the subterminal leaves. The % Term Fl indicates the number of cases in which the floral stimulus, in excess of that required for floral initiation at the subapical nodes, is translocated to the apex.

Experimental

Cestrum plants were maintained in the vegetative state for 18 months when grown in SD and for 69 days when grown in LD conditions; at the end of the experimental periods there were no signs of floral primordia upon dissection of the axillary growing points. Cestrum plants did flower, however, if they received as few as 5 LD followed by 2 SD. In plants so treated flower buds were visible within 6 days after the end of SD induction. Upon dissection of the axillary buds, flower primordia were visible within 2 days after SD induction; the first sign of the transition from the vegetative to the flowering state was the appearance of the calyx lobe primordia when the buds were 0.1 to 0.2 mm in diameter. All observations in the succeeding experiments were made 7 days after the beginning of SD induction when the buds were 0.8 to 1.0 mm in diameter and the corolla lobe primordia were visible. The development of flower buds after initiation proceeded at approximately the same rate in LD and SD and was independent of the preceding inductive treatment.

Since Cestrum continued its vegetative mode of development if all the leaves were removed before LD or SD induction, it was assumed that the leaves were the main organs responding to photoperiodic induction.

These first experiments showed that floral initiation did not begin until the plants had received both LD and SD induction, and established Cestrum as a LSDP. The remainder of the experiments are concerned with characterizing these photoperiodic requirements.

Table I

Promotion of Long-Day Induction with Supplementary Light and by Interruption of the Nyctoperiod and Inhibition of Short-Day Induction by Interruption of the Nyctoperiod and with Auxin

Treatment	% Flowering	Nodes Fl/Pl	No. of plants
A. Long-Day Induction			
1. Supplementary light			
None	0	0	8
16 hrs, 10 fc	40	1 ± 0.5	10
16 hrs, 700 fc	100	6 ± 1	8
2. Light interruption			
None	0	0	6
1 hr, 700 fc	60	3 ± 1	10
16 hrs, 700 fc	100	17 ± 2	8
B. Short-Day Induction			
1. Light interruption			
60 min, 500 fc	0	0	8
10 min, 500 fc	80	15 ± 3	5
None	100	22 ± 1	8
2. Auxin			
NAA, 10^{-4} M	57	1 ± 0.5	7
Control	100	10 ± 4	7

Long Day Induction: LD induction in LDP may be satisfied by extending a SD with low intensity light or by interrupting the NP of a SD with a short period of high intensity light and is promoted by increasing the length of the PP (10). Part of the light requirement in Cestrum could be replaced with low intensity light as was shown in the following experiment. Three groups of plants in SD conditions received either low intensity (10 fc), high intensity (700 fc), or no light throughout the 16-hr NP. After 12 days of this treatment the plants were transferred to SD conditions for 7 days. The results of the experiment (see table I, A, 1) show that supplementary low intensity light satisfied the LD requirement though it was much less effective than a high intensity light treatment of equal duration.

By interrupting the NP of a SD it was possible to satisfy LD induction although this treatment was much less effect than continuous light of equal intensity. For this experiment Cestrum plants, in SD conditions, were treated as follows for 18 days; for one group each of the 16-hr NP was interrupted after 7.5 hours with one hour of light (700 fc), a second received continuous light (700 fc) throughout the NP, and a third received an uninterrupted NP. After the 18-day period the 3 groups were transferred to SD conditions for 7 days. The results of the experiment appear in table I, A, 2.

Table II

The Critical Photoperiod for Long-Day Induction and the Critical Nyctoperiod for Short-Day Induction

Period		% Fl	% Term fl	Nodes fl/pl	No. of plants
Type	Length in hrs				
A. Long-Day Induction					
PP	11 *	0	0	0	6
PP	11.5 *	17	0	0.5 ± 0.5	6
PP	12 **	17	17	3 ± 3	6
PP	12.5 **	100	33	14 ± 2	9
PP	13 *	94	31	14 ± 2	15
PP	14 *	100	54	16 ± 3	13
PP	16 *	100	100	20 ± 1	8
B. Short-Day Induction					
NP	11 ††	0	0	0	6
NP	11.5 †	33	0	2 ± 2	6
NP	12 ††	67	0	3 ± 2	6
NP	12.5 †	67	50	13 ± 6	6
NP	13 ††	67	33	11 ± 5	6
NP	15 ††	83	17	12 ± 5	6
NP	16 †	100	33	14 ± 2	6

Conditions of temperature and light used during the LD induction in the above experiments are as follows (X = the length of the PP in hrs):

* Eight hrs in the 26° C greenhouse + X hrs of artificial light and 16 − X hrs dark at 20° C.

** X hrs of artificial light + 24 − X hrs dark at 20° C.

† Sixteen hrs of artificial light + 8 hrs at 20° C.

†† Eight hrs in the 23° C greenhouse + 8 hrs artificial light and 8 hrs dark at 17° C.

Table III

The Number of Days Required for Long-Day and Short-Day Induction

No. of days	% Fl	% Term fl	Nodes fl/pl	No. of plants
A. Long-Day Induction				
4	0	0	0	6
5	67	0	1 ± 0.5	6
7	100	17	7 ± 2	6
8	100	0	9 ± 2	6
9	100	50	17 ± 4	6
10	100	80	20 ± 3	6
18	100	100	32 ± 2	6
B. Short-Day Induction				
1	0	0	0	7
2	100	28	15 ± 4	7
3	100	43	18 ± 4	7
4	100	57	22 ± 3	7
7	100	72	23 ± 2	7

As is shown by the data in table II, A, the critical PP for LD induction is between 11 and 12.5 hours and the flowering response increases with the PP up to 16 hours. In these experiments the plants received 18 LD (the temperature and light conditions are recorded in table II, A) followed by 7 SD. In other experiments Cestrum plants were exposed to various numbers of LD followed by 7 SD. The results of one experiment, recorded in table III, A, show that a minimum of 5 days was required for LD induction and that there was a marked increase in the flowering response up to 18 LD. Throughout the LD period new leaves responding to induction were unfolded from the terminal apex and undoubtedly part of the increased flowering response above 5 LD can be attributed to this factor.

After 5 LD some of the leaves had undergone a change permitting response to SD induction and the onset of floral initiation. With increased numbers of LD there was apparently an accumulation of some substance(s) (henceforward referred to as L), permitting a greater response to SD induction. To investigate the nature of L a series of experiments were performed in which Cestrum plants were exposed to high temperatures after LD but before SD induction. If L were a protein or another heat labile substance such treatment should cause its inactivation. Two lots of plants received 15 LD followed by 7 SD; 5 hours prior to the start of the first NP of SD induction one group was placed in a darkened oven maintained at 42 ± 2° C for 2 hours and the other in a 23° C darkroom for an equivalent period. The plants were then returned to the 26° C greenhouse and 3 hours later entered the first NP of SD induction. The high temperature treatment did not damage the leaves and stems or impair subsequent vegetative development. The inhibition of floral initiation, which amounted to 82% (table IV, A), can probably be attributed to the heat lability of L.

If Cestrum plants were defoliated after LD induc-

TABLE IV

INHIBITION OF FLORAL INITIATION BY HIGH TEMPERATURE TREATMENT AFTER LONG-DAY OR SHORT-DAY INDUCTION

HEAT TREATMENT	% FL	% TERM FL	NODES FL/PL	NO. OF PLANTS
A. After Long-Day Induction				
2 hrs, 42° C ...	30	0	2 ± 1	10
Control	78	11	11 ± 2	9
B. After Short-Day Induction				
10 min, 50° C ..	86	14	8 ± 3	7
Control	100	43	18 ± 3	7

Inhibition (on the basis of Nodes Fl/Pl) is 82 % after LD induction and 66 % after SD induction.

tion, regardless of the number of LD, they remained vegetative even though new leaves developed from the terminal and axillary buds and subsequently received SD induction. If L were translocated from the leaves and accumulated in the buds or stems floral initiation should have ensued after the new leaves had received SD induction. In another experiment some leaves of a plant were exposed to LD induction and at the same time others received SD induction as follows: one lot of plants, with the exception of 3 leaves, was exposed to 25 LD (8 hours in the 23° C greenhouse plus 8 hours of artificial light and an 8-hr NP at 23° C); the 8th, 9th, and 10th leaves, counting basipetally, were covered and uncovered daily at the end and beginning, respectively, of the 8 hours in the greenhouse. In this manner the covered leaves received an 8-hr PP (SD induction) while the remainder of the plant received a 16-hr PP (LD induction). If L were translocated from the leaves receiving LD induction to those receiving SD the conditions necessary for floral initiation should have been met. However, at the end of the 25-day period none of the plants had initiated flowers. Another lot of plants was treated in the same manner for 18 days and then transferred to SD conditions for 7 days; all of these plants flowered at nodes above and below those adjacent to the SD leaves (indicating that the leaves above and below the SD leaves produced L). A third group of plants received 18 LD with none of the leaves covered and then the 8th, 9th, and 10th leaves were covered with aluminum foil, in the manner described above, for 7 days. Eighty-three % of the plants of this group flowered, demonstrating that covering the leaves with aluminum foil was sufficient to insure SD induction. The results of these experiments seemed to justify the conclusions that L was not translocated from the leaves, and, the corollary, that a leaf must receive LD before it can respond to SD induction.

SHORT DAY INDUCTION: SD induction in SDP may be inhibited by interruption of the NP with short periods of high intensity light and is promoted by increasing the length of the NP (10). For many SDP auxin applied just before or during the NP inhibits floral initiation (2) although there are exceptions (17). The results of 2 experiments with Cestrum, recorded in table I, B, 1, show the marked inhibition of SD induction by interruption of the NP. The experiments were as follows: 3 groups of plants were exposed to 15 LD followed by 2 SD; one lot received 2 uninterrupted NP and a second and a third received 10 and 60 minutes, respectively, of light (500 fc) in the middle of each NP; at the end of SD induction the plants were returned to the original LD conditions for 5 days.

The results of a single experiment, recorded in table I, B, 2, show that SD induction was severely inhibited by auxin applied prior to the NP. Two lots of plants were exposed to 18 LD followed by 2 SD. Prior to the beginning of each NP of SD induction one lot was sprayed with a 5×10^{-4} M solution of naphthalene acetic acid (NAA), adjusted to pH 7 and containing some detergent, until the solution dripped from the leaves; 20 minutes after spraying the plants were thoroughly rinsed with water and placed in the NP conditions. Another group received the same treatment except that the spray solution did not contain auxin. At the end of SD induction both lots were returned to LD conditions for 5 days.

The critical NP for SD induction was between 11 and 12 hours (table II, B) and there was a considerable increase in the flowering response with longer NP. For these experiments several lots of plants were exposed to 18 LD (temperature and light conditions recorded in table II, B) followed by SD induction for which the length of the NP was varied. The plants received 3 SD with the following light and temperature conditions: 8 hours in the 26° C greenhouse plus 16 − X hours of artificial light and an X-hr NP at 20° C. After SD induction the plants were returned to LD conditions for 4 days.

The results of one of several experiments in which the number of days for SD induction was varied (all groups received 15 LD prior to SD induction) are recorded in table III, B. A minimum of 2 SD was required for induction and there was an increased response with additional SD. Single long NP ranging from 48 to 168 hours did not satisfy the SD requirement. Since Cestrum plants survived these extended dark periods with no permanent damage to vegetative structures, they may be ideal for studying "endogenous rhythms" (4).

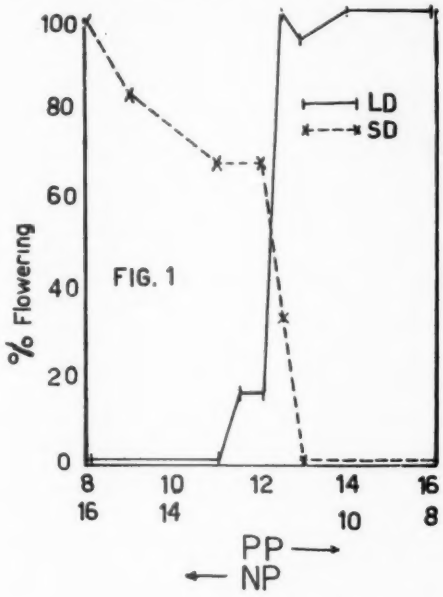
Within 4 days after the start of SD induction some of the apical and axillary buds had entered the first stages of floral differentiation. Sometime during this 4-day period the floral stimulus, henceforward referred to as S, was translocated from the leaves to the growing points. To determine the time of translocation of S, plants were defoliated at various times after the start of SD induction following the methods introduced for Xanthium (18, 8). The results of several experiments (15) demonstrated that within 8 hours after the 2nd NP of SD induction a sufficient amount of stimulus had been translocated from the leaves to cause floral initiation. The 8-hr period is

close to the minimum time for translocation of S since defoliation 5 hours after the end of the 2nd NP completely prevented floral initiation. Depending upon the conditions of the experiment, translocation of S continues for 48 to 70 hours after the 2nd NP.

A single experiment was performed to test the effect of a short period of high temperature after SD induction. Two lots of plants were exposed to 15 LD (8 hours in the 23° C greenhouse plus 8 hours of artificial light and an 8-hr NP at 23° C) followed by 2 SD. Five hours after the end of the 2nd NP one lot was placed in a darkened 50° C oven and the other in a 23° C darkroom for 10 minutes. The plants were then returned to LD conditions for 5 days. Although there was no wilting or visible damage to the leaves or growing points, a 6 cm band of phloem tissue was killed around the crown, far below the lowest leaf. If this phloem damage did not inhibit floral initiation (owing to its position the damage should not have interfered with the translocation of S), the inhibition observed was probably due to the heat lability of S. Lockhart and Hamner (11) found that the floral stimulus in Xanthium was inactivated by exposure of plants to 3 hours at 40° C at the end of the NP; if the NP were followed by 5 hours of high intensity light the floral stimulus was no longer susceptible to inactivation by the 40° C treatment. If the situation is comparable in Cestrum, it is probable that the inhibition of floral initiation was not due to the lack of time for photochemical stabilization since the plants received 5 hours of light (2000 fc) prior to the heat treatment. Nakayama (12) has shown that exposure of Pharbitis nil, a SDP, to 2 hours at 36° C severely inhibits floral initiation; in this case the inhibition may have been equivalent to that observed by Lockhart and Hamner prior to the photochemical stabilization process.

Discussion

The results of experiments recorded in tables II, A and III, A show that the flowering response depends upon the strength of LD induction. This is true even though the product of LD induction, L, is not translocated from the leaves and floral initiation does not occur until the plants have received SD induction and produced the floral stimulus, S. It is known that S is not produced in leaves which have not previously received LD induction and accumulated some quantity of L. Since the number of nodes flowering per plant, ultimately dependent upon the amount of S produced by the leaves, increases as the strength of LD induction (i.e., as the concentration of L increases), the amount of S produced must be dependent upon the concentration of L. This suggests that L is a precursor of S and required in stoichiometric rather than catalytic concentrations. The results of experiments demonstrating that high temperatures applied after LD induction inhibit floral initiation are also compatible with this suggestion. Cestrum may be an excellent plant with which to study the chemical properties of the floral stimulus or its precursor since L may be accumulated in the leaves for an extended period and released in a relatively short period of time as S.



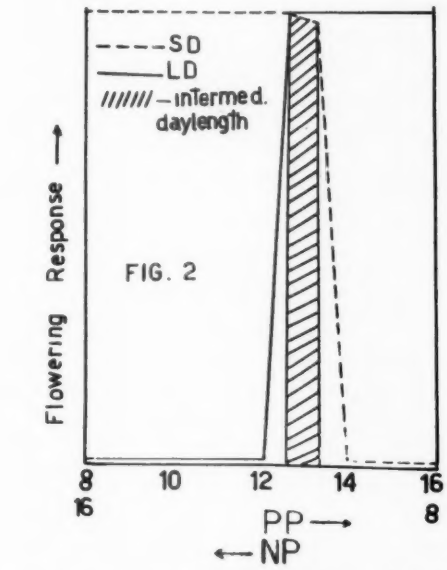


Fig. 1 (*top*). The critical PP and NP for LD and SD induction, respectively. Data are taken from table II.

Fig. 2 (*bottom*). The hypothetical curves for the PP and NP of LD and SD induction, respectively, of an intermediate day plant flowering with continuous exposure to a 13-hr PP, the region of maximum efficiency for simultaneous LD and SD induction.

The curves of figure 1, showing the relationship between the length of the PP and NP and the response to LD and SD induction, respectively, overlap in the 11.5- to 12.5-hour range. At first sight it appeared that it should have been possible to satisfy the LD and SD requirements simultaneously with continuous exposure to PP within this range. However, this was not the case and with increased exposure to the 11.5- and 12-hour PP floral initiation did not occur. When the plants were transferred from these PP to SD conditions (16-hr NP) it was noted that there was no increase in the % Term Fl with an increased number of LD as was the case with a 16-hr PP (table V). Terminal flowering indicates that S is produced in the subterminal leaves in excess of that required for floral initiation at the adjacent nodes. Since S is dependent upon L, terminal flowering is an indication of excess L produced in the subterminal leaves. With the exception of 2 plants L did not increase to excess concentrations with prolonged exposure to 11.5- or 12-hour PP, whereas it did so quickly in a 16-hr PP. Apparently the rate of production of L is much lower in the intermediate PP than in a 16-hr PP. If the rate of production decreases as the leaf age increases, this factor combined with a low starting rate may explain why L does not reach excess concentrations upon prolonged exposure to 11.5- or 12-hour PP.

(In the above discussions an arbitrary decision was made to speak of the rate of production, rather than destruction, of L; there is, however, no evidence to show that the rate of destruction is not the factor limiting the concentration of L.)

Although Cestrum is not an intermediate day plant of the type described by Allard (1), it suggests an explanation for floral initiation in plants of this category. If the intermediate day plants possessed LD and SD requirements for floral initiation and the critical PP and NP for the two reactions overlapped, including a region of high efficiency for one or both reactions, floral initiation would be possible with continuous daylength treatments within this restricted range. Figure 2 shows the hypothetical PP and NP curves of LD and SD induction, respectively, for an intermediate day plant flowering in a 13-hour PP given continuously or with LD (PP in excess of 13 hours) followed by SD (NP in excess of 11 hours). One variety of sugarcane described by Allard flowered in continuous daylength treatments only when the PP was exactly 13 hours. He did not report on experiments in which plants received first LD and then SD induction, and these are the necessary experiments to test the above model.

TABLE V

THE EFFECT OF PROLONGED LONG-DAY INDUCTION UPON THE FLOWERING RESPONSE IN INTERMEDIATE DAYLENGTHS

PHOTOPERIOD IN HRS *	NO. OF LONG DAYS **	% TERM FL	NO. OF PLANTS
11.5	18	0	6
11.5	28	0	6
11.5	40	0	6
12	18	17	6
12	28	0	6
12	42	17	6
16	7	0	7
16	14	70	13
16	18	83	21

* For all photoperiods LD induction was followed by 7 SD (8 hrs in the 26° C greenhouse and 16 hrs dark at 20° C).

** Letting X = the photoperiod in hrs, the conditions for long-day induction are X hrs artificial light + 24 − X hrs dark at 20° C.

The results of experiments involving graft unions between LDP and SDP have shown that the floral stimulus is identical in both photoperiodic types (9, 10). Grafting experiments between day neutral and photoperiodically sensitive plants indicate that the floral stimulus may be common to many, and perhaps all, plants regardless of their photoperiodic sensitivity. However, one of the difficulties in assuming the same floral stimulus in all plants is to explain how the apparently opposite light requirements of LDP and SDP result in the production of the same end product.

Borthwick et al (3) have suggested that the floral stimulus, or some precursor, is subject to destruction in the light and that this substance must reach a minimum concentration to cause floral initiation but is inhibitory at higher concentrations. On the basis of this hypothesis it was possible for the authors to explain the differences in behavior between LDP and SDP with the following argument: LDP have a relatively high rate of synthesis, or low rate of destruction, of the floral stimulus, and, conversely, SDP have a relatively low rate of synthesis of the floral stimulus. Additional light inhibits floral initiation in SDP by preventing the accumulation of a minimum concentration of the stimulus and promotes initiation in LDP by preventing the accumulation of inhibitory concentrations. The scheme proposed by Van de Sande Bakhuyzen (19, 20) is a modification of that of Borthwick et al and also assumes that the floral stimulus is the same in all plants and that the photoperiodic control of floral initiation is mediated through the photosensitized destruction of a substance common to both LDP and SDP. At the time they were proposed these hypotheses provided an explanation for all the known photoperiodic types including the intermediate day plants.

The discovery of the LSDP by Resende (13) suggests that a modification of these theories is required. His most recent findings (14) and those reported in this paper for Cestrum, another LSDP; indicate that the LD and SD requirements for this group are the same as those for LDP and SDP, respectively. If they are the same, these results suggest that the product of LD induction in LDP differs from that of SD induction in SDP, perhaps in the same way that L differs from S in Cestrum.

Gott et al (5) have shown that unvernalized winter rye behaves as a SDP as well as a LDP in that

floral initiation is promoted by SD preceding LD. In his review, Lang reported some additional cases in which the vernalization requirement of winter cereals was replaced by SD induction, and Wellensiek et al (21) have reported the same behavior for *Campanula medium*, calling it an SLDP.

There is no data demonstrating the identity of the floral stimulus or of the pigment receptor of the photosensitized reactions between the LDP and SDP on the one hand and the LSDP and SLDP on the other. With this information, which is basic for constructing a generalized scheme, the most perplexing problem, that of the relationship between the photosensitized reactions and the synthesis of the floral stimulus, will remain unsolved.

Summary

1. *Cestrum nocturnum* was shown to be a LSDP, that is, it required LD followed by SD induction for floral initiation. LD induction was promoted by supplementary low intensity light and by interruption of long NP with short periods of high intensity light. SD induction was inhibited by interruption of a NP with a short period of light and by auxin applied before the start of a NP. Thus both the LD and SD requirements are comparable to those of LDP and SDP, respectively.

2. A minimum of 5 LD was required for LD induction and the critical PP was between 11 and 12.5 hours. A minimum of 2 SD was required for SD induction and the critical NP was between 11 and 12 hours.

3. Only leaves which had previously received LD induction responded to SD induction suggesting that the product of LD induction was not translocated from the leaves. The product of SD induction was translocated to the growing points shortly after the end of the second NP.

4. The product of LD induction was probably heat labile. The same was indicated for the product of SD induction although the experiment was equivocal due to vegetative damage accompanying the inhibition of floral initiation.

5. With Cestrum as a model it was suggested that floral initiation in the intermediate day plants may be controlled by two overlapping photoperiodic reactions.

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Literature Cited

1. Allard, H. A. Complete or partial inhibition of flowering in certain plants when days are too long or too short. Jour. Agr. Research 57: 775–789. 1938.
2. Bonner, J. and Liverman, J. Hormonal control of floral initiation. In: Growth and Differentiation of Plants, W. E. Loomis, ed. Pp. 283–303. Iowa State College Press, Ames, Iowa 1953.
3. Borthwick, H. A., Parker, M. W. and Hendricks, S. B. Wavelength dependence and the nature of photoperiodism. In: Vernalization and Photoperiodism, A. E. Murneek and R. O. Whyte, ed. Pp. 71–78. Chronica Botanica Co., Waltham, Massachusetts 1948.
4. Bünning, E. Morphogenesis in plants. In: Survey of Biological Progress, Vol. II, G. Avery, ed. Pp. 105–140. Academic Press, Inc., New York 1952.
5. Gott, M. B., Gregory, F. G. and Purvis, O. N. Studies in vernalization of cereals. XIII. Photoperiodic control of stages in flowering between initiation and ear formation in vernalized and unvernalized Petkus winter rye. Ann. Bot. N.S. 21: 87–126. 1955.
6. Garner, W. W. and Allard, H. A. Effect of the relative length of day and night and other factors of the environment on growth and reproduction in plants. Jour. Agr. Research 18: 553–606. 1920.
7. Hamner, K. C. Interrelation of light and darkness in photoperiodic induction. Bot. Gaz. 100: 658–687. 1940.
8. Khudairi, A. and Hamner, K. C. The relative sensitivity of Xanthium leaves of different ages to photoperiodic induction. Plant Physiol. 29: 251–257. 1954.
9. Khudairi, A. and Lang, A. Flowering hormone of short day and long day plants. Congr. intern. bot. Rapports et Communications aux Sections 11 et 12. P. 331. 8th Congr., Paris 1954.
10. Lang, A. Physiology of flowering. Ann. Rev. Plant Physiol. 3: 265–306. 1952.
11. Lockhart, J. A. and Hamner, K. C. Partial reactions in the formation of the floral stimulus in Xanthium. Plant Physiol. 29: 509–513. 1954.
12. Nakayama, S. Experimental researches on photoperiodism. (2). Analysis of the mechanism of the dark reaction by the high temperature interruption. Bull. Soc. Plant Ecol. (Japan) 3: 95–107. 1953.
13. Resende, F. Long-short day plants. Portugaliae Acta Biol. Serie A 3: 318–321. 1952.
14. Resende, F. Acerca do impulso em floral plantas de "dia longo-corto" e plantas de "dia curto-longo." Rev. fac. cienc., Lisboa, Ser. 2C 3: 447–534. 1953.
15. Sachs, R. M. Floral initiation in *Cestrum nocturnum*. Doctoral thesis, California Institute of Technology, Pasadena, California 1955.
16. Schulz, O. E. Solanacearum genera nonnulla. In: Symbolae Antillanae sen Fundamenta Floral Indiae Occidentalis, I. Urban, ed. Vol. 6, chap. 5. Pp. 254–255. Borntraeger, Leipzig. 1909.
17. Sen Gupta, J. C. and Guhathakurta, P. Effect of IAA and TIBA treatments on the induction of flowering. Congr. intern. bot. Rapports et Communications aux Sections 11 et 12. P. 332. 8th Congr., Paris 1954.
18. Skok, J. and Scully, N. J. Characteristics and movement of the flowering stimulus from the induced leaf of Xanthium. Bot. Gaz. 116: 142–147. 1954.
19. Van de Sande Bakhuyzen, H. L. Flowering and flowering hormones (one single scheme for both long day and short day plants). II.A. Photoperiodism in long day plants. Proc. Koninkl. Ned. Akad. Wetenschap. C. 56: 603–623. 1951.

20. Van de Sande Bakhuyzen, H. L. Flowering and flowering hormones (one single scheme for long day and short day plants). III.A. Photoperiodism in short day plants. Proc. Koninkl. Ned. Akad. Wetenschap. C. 56: 164–184. 1953.

21. Wellensiek, S. J., Doorenbos, J. and de Zeeuw D. The mechanism of photoperiodism. Congr. intern. bot. Rapports et Communications aux Sections 11 et 12. P. 332. 8th Congr., Paris 1954.

22. Went, F. W. The Earhart Plant Research Laboratory. Chronica Botanica 12: 89–108. 1950.

THE CONVERSION OF $C^{14}O_2$ AND CH_3-$C^{14}O$-COOH TO CITRIC AND MALIC ACIDS IN THE TOMATO FRUITS[1,2]

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Recent studies have shown that following injection of acetate-1-C^{14}, into mature green tomatoes, radioactivity appears in the respiratory CO_2, protein, organic acids and certain other fractions isolated from the homogenized tomatoes (27). Degradation of citric and malic acids isolated therefrom indicated the active operation of the tricarboxylic acid cycle as the major pathway of acetate metabolism and the biosynthesis of citric acid.

To trace the mode of biosynthesis of C_4 acids, further experiments have been carried out in this laboratory using green tomatoes in the dark with $C^{14}O_2$ and $CH_3C^{14}OCOOH$ as test substrates, respectively. Data from degradation studies on citric and malic acids are presented which reveal that although the $C_3 + C_1$-type condensation is present in this fruit, it is probably not the major pathway for the biosynthesis of malic acid.

Materials and Methods

Introduction of Labeled Compounds: Michigan State Forcing tomatoes grown in a greenhouse and picked at the mature green state (6 fruits, 657 gm) were placed in a desiccator under slightly reduced pressure and exposed to 1 mc of $C^{14}O_2$ (specific activity 9.27×10^8 cpm/millimole) which was generated inside the desiccator by injecting 20 % perchloric acid into a small vial containing $BaC^{14}O_3$. The tomatoes were kept in the dark at 24° C for 48 hours after which time the system was swept with CO_2-free air and any residual $C^{14}O_2$ was trapped in CO_2-free NaOH.

In a second experiment, 0.134 millimole of CH_3C^{14}-OCOOH (specific activity 1.02×10^8 cpm/millimole) was dissolved in 3.5 ml of water and injected into the locules of 5 tomatoes (total weight of 987 gm). The fruits were placed in a large desiccator kept in the dark. The respiratory CO_2 was swept continuously from the system with CO_2-free air and collected in NaOH solution. The trap solution was replaced periodically with fresh CO_2-free NaOH. Carbonate in the solution was recovered as $BaCO_3$ for radioactive assay.

The specific activity of the respiratory CO_2 reached a maximum in approximately 15 hours (fig 1). To insure a steady state in the incorporation of radioactivity into various compounds in the fruit, the experiment was terminated after 46 hours at which time the cumulative activity curve of the respiratory CO_2 had leveled off as shown in figure 1.

Isolation of Fruit Acids: The fruit from the CO_2 fixation experiment was immediately sliced and homogenized in a Waring blendor. After the pulp was separated from the juice by centrifugation, washed successively with water, ethanol and ethyl ether, and dried in vacuo, the combination of aqueous washings and juice was then passed through an IR-120 ion exchange column to remove the amino acids. Next the effluent was concentrated and passed through an IRA-400 resin column, and the organic acids were recovered from the IRA-400 resin by means of HCl elution and exhaustive ether extraction of the concentrated eluate. Finally upon removal of ether, the mixed acids were redissolved in water.

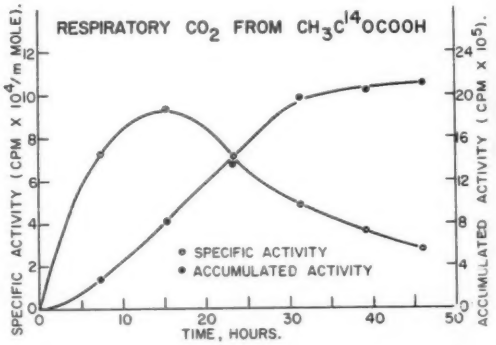

Fig. 1. Accumulative and specific activity in respiratory CO_2 of tomato fruits utilizing CH_3-$C^{14}O$-COOH.

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[2] This research was supported by Contract No. AT(45-1)-573 from the Atomic Energy Commission. Published with the approval of Monographs Publication Committee, Research paper No. 291, School of Science, Department of Chemistry. A portion of the work described here is taken from the thesis presented by Donald R. Buhler for the degree of Doctor of Philosophy at Oregon State College, 1956.

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In the pyruvic acid experiment, the procedure for isolation of the fruit acids was subsequently modified because of the detection of interaction of fruit sugars with the strongly basic ion exchange resin IRA-400 (5). The combined juice and aqueous washings was put through an IR-120 resin column, and the effluent was concentrated, acidified with sulfuric acid and subjected to exhaustive ether extraction. The ether was removed from the combined extracts and the residual mixed acids were redissolved in water.

A portion of the aqueous solution of mixed acids was taken to dryness over phosphorus pentoxide in vacuo, acidified with sulfuric acid and subjected to silica gel column chromatography according to the method of Bulen et al (6). The identity and purity of all fractions were established by paper chromatography (4, 11) and radioautography. The content of acids in the original mixed acids fraction and pure fractions obtained in the chromatographic separation was estimated by colorimetric methods (17, 22).

Malic acid was purified through its lead salt with subsequent removal of lead ion with H_2S. Inert carrier was then added and the diluted malic acid was further purified by recrystallization from a solution of dry benzene and acetone.

Citric acid was isolated as its calcium salt after appropriate dilution with inert carrier acid.

Degradation Procedures: The total activity of malic acid was determined by dry combustion to carbon dioxide. A portion of the acid was decarbonated with concentrated sulfuric acid according to the method of Racusen and Aronoff (18) and the CO so formed was converted to CO_2 for the specific activity of C-1. Finally, a sample was oxidized with potassium permanganate according to the procedure of Wood et al (29) to give carbon dioxide from C-1, 5 and acetaldehyde (corresponding to C-2, 3). The aldehyde was converted to iodoform which corresponds to the activity of C-3 and the activity of C-4 was obtained by difference.

The molar specific activity of the citric acid was obtained either by dry combustion or by persulfate oxidation. A second sample of citric acid was treated with concentrated sulfuric acid according to the Martin and Wilson modification (13) of the method of Weinhouse, Medes and Floyd (28) to yield CO and CO_2 from C-6 and C-1, 5 respectively. The acetone obtained in the same reaction was oxidized subsequently by the wet combustion method of Van Slyke and Folch (24) to CO_2. The activity of the latter is equivalent to that of C-2, 3, 4.

Acetone was obtained also from C-2, 3, 4 as mercury-acetone complex by the dichromate oxidation method of Lewis and Weinhouse (10). The mercury-acetone complex as converted to iodoform which gave acetone complex was converted to iodoform which gave the specific activity of C-2, 4 directly and C-3 by difference; a portion of the Deniges complex was also oxidized to carbon dioxide for the total activity of C-2, 3, 4. As a further verification of the degradation results, citric acid was also degraded by the method of Martin, Wilson and Burris (12) to give the total activity in C-1, 5, 6. The pentabromoacetone from the three central carbon atoms was converted to carbon dioxide, which corresponds to the sum of C-2, 3, 4.

All samples were counted as barium carbonate in the conventional manner to a standard error of 2 % and corrected for background and self-absorption.

Result and Discussion

The distribution of the incorporated activity in various crude fractions of tomatoes as derived from $CH_3C^{14}OCOOH$ and $C^{14}O_2$ is given in table I. The incorporated C^{14} activity from either carbon source was found in the organic acids and less noticeably in the amino acids fractions.

The isotopic distribution patterns of malic and citric acids isolated from both experiments are given in table II. In view of the difference in the quantity of acids present in the fruits, the labeling level of various carbon atoms of these acids was corrected for the pool size of each acid in the fruit used in the present experiments. The corrected specific activities as well as the pool size of malic and citric acids are given in table III.

From $C^{14}O_2$, as expected, one finds the activity located exclusively in the carboxyl groups of the fruit acids. The considerably heavier labeling in C-4 of malic acid as compared to that of C-1, indicates that the mechanism of CO_2 fixation in tomatoes belongs to the Wood-Werkman (29) or malic enzyme (15) type. It is interesting to note that the partial randomization of the malic acid carboxyl groups observed here (probably as a result of equilibration with fumarate) is in contrast to the recent report on CO_2 fixation in apples (1, 2) where complete randomization was realized. In citric acid, the tertiary carboxyl carbon was significantly more heavily labeled than the primary carboxyl carbon atoms. By assuming that (a) C-5 of citric acid is unlabeled as shown by the absence of $C^{14}O_2$ activity in the fat fraction (table I), hence the "acetate" unit; and that (b) the C_4 condensing component in the citric acid synthesis is symmetrical as indicated by the equal labeling of C-2 and

Table I

Distribution of C^{14} Activity in Fractions Isolated from Tomato Fruit

Fraction	CO_2 experiment		Pyruvate experiment	
	Sp. activity (cpm/millimole)	Total activity (cpm)	Sp. activity (cpm/millimole)	Total activity (cpm)
Activity administered	9.27×10^8	1.4×10^8	1.02×10^8	1.37×10^7
Respiratory CO_2	4.26×10^6	3.17×10^7	6.32×10^4	2.11×10^6
Organic acids	....	8.95×10^6	....	1.92×10^6
Amino acids	....	1.1×10^6	....	0.40×10^6
Sugars	....	0.01×10^6	....	0.26×10^6
Fat	....	0	....	....

TABLE II

ISOTOPIC DISTRIBUTION PATTERN IN MALIC AND CITRIC ACIDS IN TOMATOES UTILIZING $CH_3C^{14}OCOOH$ AND $C^{14}O_2$

CARBON ATOM	$C^{14}O_2$ EXPERIMENT		$CH_3C^{14}OCOOH$ EXPERIMENT	
	SP. ACTIVITY (CPM $\times 10^5$/MILLIMOLE)	% TOTAL	SP. ACTIVITY (CPM $\times 10^4$/MILLIMOLE)	% TOTAL
	Malic Acid			
C-1	24.9	35	23.7	40.0
C-2	0	0	4.5	7.7
C-3	0	0	2.0	3.4
C-4	40.1	57	26.8	46.0
Whole molecule	70.5	100	58.7	100.0
	Citric Acid			
C-1 + C-5	12.2	43	6.97	87.0
C-2	0	0	0.13	1.6
C-3	0	0	0.13	1.6
C-4	0	0	0 *	0
C-6	15.3	54	1.32	16.4
Whole molecule	28.4	100	8.04	100.0

* Based on theoretical consideration.

C-3 of citric acid in the pyruvate experiment, one could then assign the activity of primary carboxyl carbon atoms to C-1 alone and expect to find equal labeling in C-1 and C-6 of citric acid. The observed excessive labeling in the tertiary carboxyl carbon atom consequently reflects the occurrence of some other CO_2 incorporation mechanism such as the formation of isocitrate from alpha-ketoglutarate and CO_2 (3, 8, 14).

TABLE III

DISTRIBUTION OF RADIOACTIVITY IN CITRIC AND MALIC ACIDS FROM TOMATO FRUITS UTILIZING $CH_3C^{14}OCOOH$ AND $C^{14}O_2$ CORRECTED FOR POOL SIZE

CARBON ATOM	$C^{14}O_2$ EXPERIMENT * (SP. ACTIVITY—CPM $\times 10^5$/MILLIMOLE)	$CH_3C^{14}OCOOH$ EXPERIMENT ** (SP. ACTIVITY—CPM $\times 10^5$/MILLIMOLE)
	Malic Acid	
Whole molecule	4.6	1.4
C-1	1.6	0.5
C-2	0	0.1
C-3	0	0.05
C-4	2.6	0.6
	Citric Acid	
Whole molecule	4.4	1.2
C-1 and C-5	1.9	1.1
C-2	0	0.02
C-3	0	0.02
C-4	0	0 †
C-6	2.3	0.2

* In the fruit 6.5 millmoles malic acid and 15.3 millimoles citric acid were found.

** In the fruit 2.3 millimoles malic acid and 15.2 millimoles citric acid were found.

† Assumed to be unlabeled.

Since the demonstration of the occurrence of $C_1 + C_3$ condensation (with CO_2) in tomatoes does not necessarily imply the quantitative significance of this reaction in the overall acid biosynthesis, the pyruvate experiment was therefore carried out. Pyruvate-2-C^{14}, a key intermediate in the glycolytic scheme, is capable of giving rise to C_4 acid with labeling mainly in C-2 and C-3 by way of CO_2 fixation. On the other hand, this compound can also be converted to acetate which is in turn used to synthesize a C_4 acid through Thunberg-type condensation (23) with labeling confined to C-1 and C-4. Comparison of the specific activity of the C-2 and C-3 with that of C-1 and C-4 will then provide a direct indication of the extent of the operation of the $C_3 + C_1$ reaction. Thus, in yeast which was utilizing pyruvate-2-C^{14} as sole carbon source, Wang et al (25) found 62 % of the activity in C-2 and C-3 of aspartic acid and concluded that the $C_3 + C_1$ type reaction is responsible for the metabolism of as much as 70 % of the available pyruvate in the yeast.

In the present experiment, the detection of labeling in the noncarboxyl carbon atoms of malic acid and citric acid substantiates the fact that CO_2 fixation occurs in this fruit. However, the presence of only 11 % of the C^{14} activity in the C-2, 3 of malic acid indicates that CO_2 fixation is probably not a major pathway in the biosynthesis of malic acid in tomatoes. Judging from the specific activity curve of the respiratory CO_2, (fig 1) it is reasonable to believe that a steady state in labeling had been reached at the time the pyruvate experiment was terminated; yet the specific activity of malic acid was higher than that of the malic acid skeleton in citric acid even after pool size is taken into consideration (table III). This fact implies that malic acid was synthesized from pyruvate prior to citric acid through a pathway unrelated to the citric acid cycle. Although the nature of this pathway cannot be defined in the present work, it is possible that condensation of two C_2 units of the Thunberg type might play an important role in the overall acid biosynthesis in this fruit. In *Aspergillus niger*, as much as 40 % of the citric acid was reported to originate through the C_2-C_2 condensation pathway (19).

The isotopic distribution pattern of citric acid in the pyruvate experiment is in good agreement with that of malic acid. Thus, if one assumes that C-1, 2, 3, 6 in citric acid are derived from the carbon skeleton of malic acid by way of the conventional citric acid biosynthesis (27), the activity of C-1, 6 and of C-2, 3 will then be 9 % and 91 %, respectively, of the four-carbon fragment in citric acid. These figures compare favorably with 11 % and 86 %, respectively, observed in malic acid.

It is also interesting to note that C-2 and C-3 of citric acid are approximately equal in activity. Since these two carbon atoms are equivalent to C-2 and C-3 of the C_4 condensing component in the classical citric acid biosynthesis, it is therefore indicated that the C_4

compound must be symmetrical in respect to its labeling pattern. In view of the fact that malic acid isolated from the same fruit is not symmetrically labeled it is unlikely that oxalacetate could have been the condensing partner. This is true since randomization of the latter by equilibration with a symmetrical C_4 acid (such as fumaric acid) would have involved malic acid as an intermediate. It thus appears that a symmetrical C_4 acid such as fumaric or succinic acid is probably participating in the citric acid formation in tomato fruits.

Summary

1. The intramolecular distribution of radioactivity has been studied in citric and malic acids isolated from mature green tomatoes treated with $C^{14}O_2$ or $CH_3C^{14}OCOOH$.

2. Carbon dioxide fixation appeared not to be the major pathway for acid synthesis in tomato fruit in the dark.

3. Evidence on the incorporation of $C^{14}O_2$ activity into the tertiary carboxyl carbon atom of citric acid is presented.

Literature Cited

1. Allentoff, N., Phillips, W. R. and Johnston, F. B. A C^{14} study of carbon dioxide fixation in the apple. I. The distribution of incorporated C^{14} in the detached MacIntosh apple. Jour. Sci. Food Agr. 5: 231–234. 1954.
2. Allentoff, N., Phillips, W. R. and Johnston, F. B. A C^{14} study of carbon dioxide fixation in the apple. II. Rates of carbon dioxide fixation in the detached MacIntosh apple. Jour. Sci. Food Agr. 5: 234–238. 1954.
3. Barban, S. and Ajl, S. J. Interconversion of isocitrate and α-ketoglutarate by *Escherichia freundii*. Jour. Bacteriol. 66: 68–71. 1953.
4. Buch, M. C., Montgomery, R. and Porter, W. L. Identification of organic acids on paper chromatograms. Anal. Chem. 24: 489–491. 1952.
5. Buhler, D. R., Thomas, R. C., Christensen, B. E. and Wang, C. H. Epimerization and fragmentation of glucose by quaternary ammonium base type anion exchange resins. Jour. Amer. Chem. Soc. 77: 481–482. 1955.
6. Bulen, W. A., Varner, E. and Burell, R. C. Separation of organic acids from plant tissues. Anal. Chem. 24: 187–190. 1952.
7. Campbell, J. J. R., Smith, R. A. and Eagles, B. A. A deviation from the conventional tricarboxylic acid cycle in *Pseudomonas aeruginosa*. Biochim. Biophys. Acta 11: 594. 1953.
8. Grisolia, S. and Vennesland, B. Carbon dioxide fixation in isocitric acid. Jour. Biol. Chem. 170: 461–465. 1947.
9. Harary, I., Korey, S. R. and Ochoa, S. Biosynthesis of dicarboxylic acids by carbon dioxide fixation. Jour. Biol. Chem. 203: 595–604. 1953.
10. Lewis, K. B. and Weinhouse, S. Studies on the mechanism of citric acid production in *Aspergillus niger*. Jour. Amer. Chem. Soc. 73: 2500–2503. 1951.
11. Lugg, J. W. H. and Overell, B. T. One- and two-dimensional partition chromatographic separation of organic acids on an inert sheet support. Australian Jour. Sci. Research 1A: 98–111. 1948.
12. Martin, S. M., Wilson, P. W. and Burris, R. H. Citric acid formation from $C^{14}O_2$ by *Aspergillus niger*. Arch. Biochem. 26: 103–111. 1950.
13. Martin, S. M. and Wilson, P. W. Uptake of $C^{14}O_2$ by *Aspergillus niger* in the formation of citric acid. Arch. Biochem. Biophys. 32: 150–157. 1951.
14. Ochoa, S. J. Biosynthesis of tricarboxylic acids by carbon dioxide fixation. III. Enzymatic mechanisms. Jour. Biol. Chem. 174: 133–157. 1948.
15. Ochoa, S., Mehler, A. H. and Kornberg, A. Biosynthesis of dicarboxylic acids by carbon dioxide fixation. I. Isolation and properties of an enzyme from pigeon liver catalyzing the reversible oxidative decarboxylation of l-malic acid. Jour. Biol. Chem. 174: 979–1000. 1948.
16. Olson, J. A. D-Isocitric lyase system: The formation of glyoxylic and succinic acids from D-isocitric acid. Nature 174: 695–696. 1954.
17. Owens, H. S., Goodban, A. E. and Stark, J. B. Fractionation of organic acids in sugar beets by ion exchange resins. Anal. Chem. 25: 1507–1511. 1953.
18. Recusen, D. W. and Aronoff, S. Metabolism of soybean leaves. V. The dark reaction following photosynthesis. Arch. Biochem. Biophys. 42: 25–40. 1952.
19. Shu, P., Funk, A. and Neish, A. C. Mechanism of ctiric acid formation from glucose by *Aspergillus niger*. Canadian Jour. Biochem. Physiol. 32: 68–80. 1954.
20. Smith, R. A. and Gunsalus, I. C. Isocitritase: a new tricarboxylic acid cleavage system. Jour. Amer. Chem. Soc. 76: 5002–5003. 1954.
21. Smith, R. A. and Gunsalus, I. C. An alternate route of tricarboxylic acid formation. Bacteriol. Proc. 1955: 114. 1955.
22. Speck, J. F., Moulder, J. W. and Evans, E. A. The biochemistry of the malaria parasite. V. Mechanism of pyruvate oxidation in the malaria parasite. Jour. Biol. Chem. 117: 119–144. 1946.
23. Thunberg, T. Intermediary metabolism and the enzymes concerned therein. Skand. Arch. Physiol. 40: 1–91. 1920.
24. Van Slyke, D. D. and Folch, J. Manometric carbon determination. Jour. Biol. Chem. 136: 507–541. 1940.
25. Wang, C. H., Thomas, R. C., Cheldelin, V. H. and Christensen, B. E. Conversion of acetate and pyruvate to aspartic acid in yeast. Jour. Biol. Chem. 197: 663–667. 1952.
26. Wang, C. H., Christensen, B. E. and Cheldelin, V. N. Conversion of acetate and pyruvate to g'utamic acid in yeast. Jour. Biol. Chem. 201: 683–688. 1953.
27. Wang, C. H., Hansen, E. and Christensen, B. E. Conversion of C^{14}-labeled acetate to citric and malic acids in the tomato fruit. Plant Physiol. 28: 741–745. 1953.
28. Weinhouse, S., Medes, G. and Floyd, N. F. Fatty acid metabolism. V. The conversion of fatty acid intermediates to citrate, studied with the aid of isotopic carbon. Jour. Biol. Chem. 166: 691–703. 1946.
29. Wood, H. G., Werkman, C. H., Hemingway, A. and Nier, A. O. The position of carbon dioxide carbon in succinic acid synthesis of heterotrophic bacteria. Jour. Biol. Chem. 139: 377–388. 1941.

RELATION OF PHOSPHORUS NUTRITION TO THE TRANSLOCATION OF 2,4-DICHLOROPHENOXYACETIC ACID IN TOMATO PLANTS[1,2]

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It has frequently been observed that the effectiveness of such herbicides as 2,4-dichlorophenoxyacetic acid (2,4-D) may vary from season to season or with different habitats even when the same species of plant is being treated. One of the factors which is likely to be different under such conditions is the availability of minerals to the plants. Since many metabolic processes are affected by different conditions of mineral nutrition, the writers have been conducting experiments on the relation of several types of mineral deficiency to the movement of 2,4-D in plants. Experiments dealing with such effects of phosphorus deficiency in the tomato plant are described in this paper.

EXPERIMENTATION AND RESULTS

Tomato seeds (Stone variety) were planted in quartz sand in 4-inch glazed soil jars in the greenhouse and were watered with a nutrient solution which contained no phosphorus. The plants were thinned periodically until 3 uniform plants remained in each pot. The plants exhibited severe phosphorus deficiency symptoms by the time they were 30 days old. At this time they were divided into 4 series with 34 pots (102 plants) in each series. Each series was divided into 2 groups one of which was to be treated with 2,4-D and the other not so treated. By watering them with a complete nutrient solution, the plants in series A were given phosphorus three days before 2,4-D was applied to the treated group; the plants in series B were given phosphorus 8 hours before the time of applying the 2,4-D; those in series C received phosphorus at the time of applying the 2,4-D; and those in series D were watered with the minus phosphorus solution until 24 hours after the growth regulator was applied to the treated group and were then given the complete solution. By use of a micropipette, 0.025 ml of 60 % ethanol containing 100 μgm of 2,4-D and 0.5 % Carbowax 1500 was applied as a drop usually to the first true leaf of each plant to be treated. In some cases the first leaves were quite severely injured, apparently as a result of the phosphorus deficiency, and the 2,4-D was applied to the second or third leaf. Ten plants in each series were treated with 2,4-D which was labeled in the carboxyl position with C^{14}. The plants were treated at approximately 2:30 P.M. and no supplementary illumination was used. The curvature of the stems was measured with a transparent protractor 20 hours after the application of the growth regulator. The results of these measurements appear in table I.

Immediately after measuring the curvatures, the 10 plants in each series which had received the C^{14}-labeled 2,4-D were harvested. The roots of these plants were washed free from the sand in which they were growing and any unabsorbed 2,4-D was washed off the treated leaves with a small stream of 70 % ethanol followed by distilled water. The treated leaf was then severed from the rest of the plant at the

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TABLE I

RELATION OF TIME OF ADDING PHOSPHORUS TO THE EFFECT OF 2,4-D ON PHOSPHORUS DEFICIENT TOMATO PLANTS

SERIES	TIME OF ADDING P IN RELATION TO TIME OF APPLICATION OF 2,4-D	MEAN STEM CURVATURES OF TREATED PLANTS 20 HRS AFTER TREATMENT WITH 2,4-D *		MEAN FRESH WTS OF TOPS 21 DAYS AFTER TREATMENT WITH 2,4-D			
				TREATED WITH 2,4-D		CONTROLS, NO 2,4-D	
		NO. OF PLANTS	DEGREES CURVATURE WITH STD ERROR **	NO. OF PLANTS	WTS (MG) WITH STD ERROR †	NO. OF PLANTS	WTS (MG) WITH STD ERROR ††
A	3 Days before	59	42 ± 3.8	38	73 ± 14	39	785 ± 113
B	8 Hrs before	50	21 ± 1.4	39	182 ± 57	38	317 ± 50
C	0 Hrs before	48	20 ± 2.2	38	140 ± 47	38	353 ± 83
D	24 Hrs after	38	15 ± 1.4	28	267 ± 38	34	340 ± 50

* There were minor curvatures, random as to direction, among the untreated control plants. Since the mean of these was well below 5° they were not recorded.

** Curvature in Series A is significantly greater than that of each of the other series at the 0.01 level. Curvature in Series B is significantly greater than that of Series D at the 0.01 level.

† Weights of treated plants in Series D are significantly greater than those in Series A at 0.01 level and significantly greater than those in Series C at 0.05 level.

†† Weights of treated plants are significantly less than those of control plants in Series A at 0.01 level and in Series C at 0.05 level.

base of the petiole, the stem was cut in two just above the cotyledonary node, and the hypocotyl was cut near its center. The plants were segmented in this manner to avoid the possibility of movement of radioactive material through the plant subsequent to harvesting. The 4 segments of each of these plants were then placed between pieces of filter paper in a plant press and dried in a vacuum oven at 60° C. When thoroughly dry these plants were removed from the press and each was mounted on a small piece of herbarium paper. The mounted plants were then placed on Ansco Non-Screen X-ray film in the dark and were left there for 30 days after which time the film was developed. Prints of the autoradiograms are shown in figure 1.

Also at 24 hours after application of the growth regulator, the treated leaves were removed from all of the plants which had received unlabeled 2,4-D and a

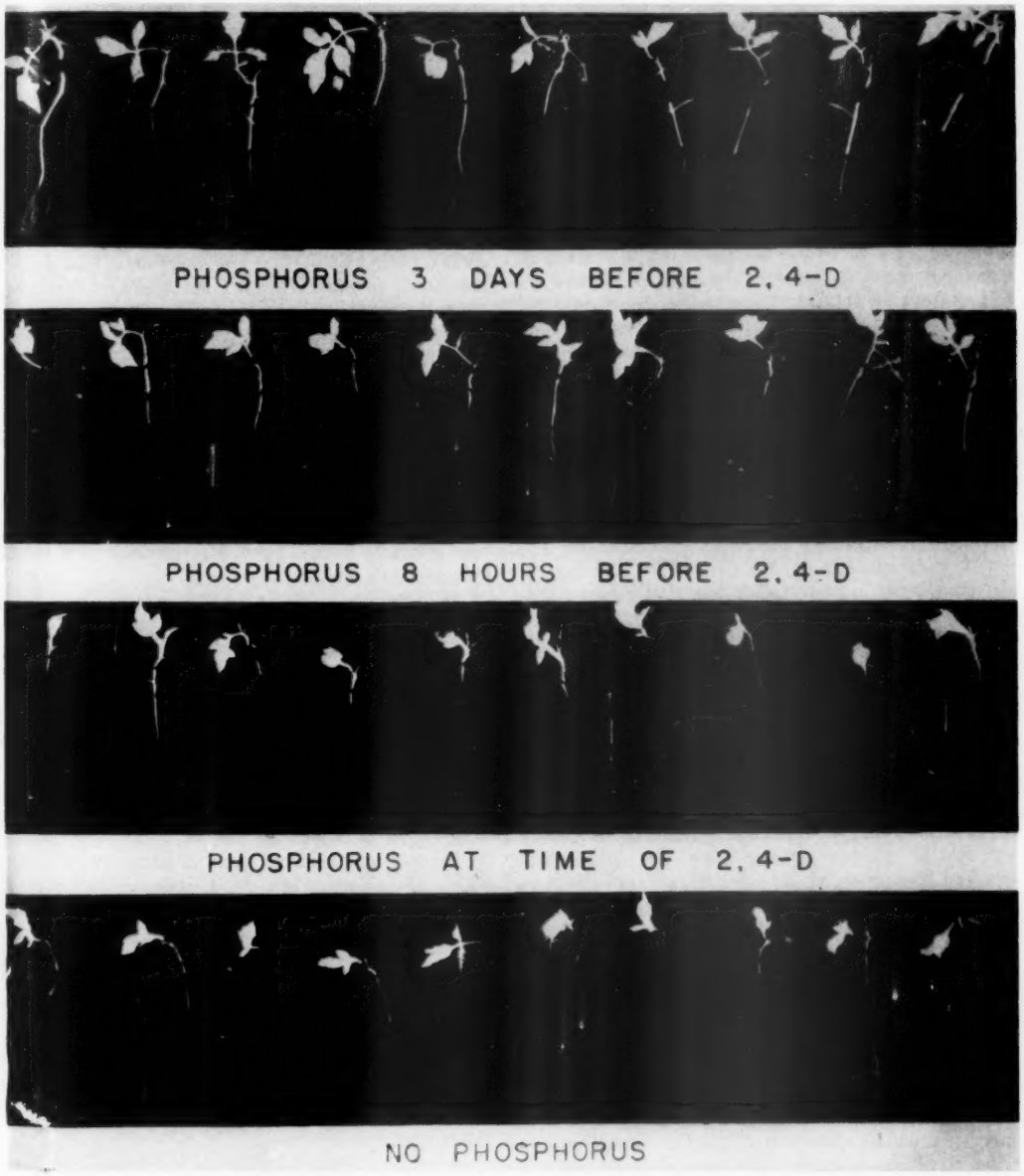

FIG. 1. Autoradiograms of P-deficient tomato plants which were supplied with P at different time intervals before being treated with C^{14}-labeled 2,4-D.

similar leaf was removed from each plant which had received no 2,4-D. The growth of these plants was observed for 21 days after application of the growth regulator. At the end of this time the plants were cut off at the sand level and their individual fresh weights determined. The weights are shown in table I.

Two other similar experiments have been performed. In both of these the results were similar to those described above.

Discussion

The data shown in table I indicate that the application of phosphorus before treatment of phosphorus deficient tomato plants with 2,4-D enhanced the effect of the growth regulator. When phosphorus was supplied to the roots of phosphorus deficient plants 3 days before 2,4-D was applied to the leaves, significantly greater stem curvatures and inhibition of growth of the terminal bud occurred than when the supply of phosphorus was delayed until 24 hours after treatment with 2,4-D. Also the stem curvatures resulting from 2,4-D treatment were significantly greater when the plants received phosphorus 8 hours before treatment than when no phosphorus was supplied until 24 hours after treatment. Even applying phosphorus at the time of application of the 2,4-D resulted in significantly less fresh weight of the tops and in greater stem curvature than was obtained when no phosphorus was supplied until 24 hours after treatment.

Stem curvature has been used as evidence of the translocation of growth regulating substances by several investigators (2, 9, 11, 13). The inhibition of the growth of the terminal buds and trifoliate leaves also has been used as evidence of this process in bean plants (11, 13). We believe that both stem curvature and inhibition of the growth of tops are fairly reliable indices for the measurement of translocation of 2,4-D in tomato plants.

The autoradiograms (fig 1) indicate that little of the C^{14} reached the lower parts of the hypocotyls and roots of the plants which did not receive phosphorus before the labeled 2,4-D was applied to them. A considerable amount of radioactive material did move through the stems and hypocotyls of the plants which received phosphorus 3 days before the application of the growth regulator and in 8 out of the 10 plants the radioactivity was detected in the roots. Such movement appears to have been greater in the group which received the phosphorus 8 hours before application of the 2,4-D than in either of the groups which received the phosphorus later but less than in the group which received the phosphorus 3 days before. There is considerable evidence that 2,4-D is metabolized in the plant (4, 5, 15), and it cannot be said that the movement of the radioactive material in these cases is definite proof of the movement of 2,4-D. Since the data from the autoradiograms seem to agree with the evidence based on such growth responses as stem curvature and inhibition of terminal growth, we believe it likely that at least some of the C^{14} has remained attached to a biologically active form of the growth regulator, at least for the 24-hour period tested in these experiments.

That the presence of translocatable food materials is required for the translocation of exogenous growth regulators seems well established (7, 9, 11, 12, 13, 14). Gauch and Dugger (3) have demonstrated that boron deficiency results in a reduction in the rate of translocation of sugar, and Mitchell et al (10) found that the addition of boron greatly augmented the effect of sugar in increasing the movement of several growth regulators through bean plants. They believed this effect of boron was due to its effect in increasing the rate of translocation of sugar. No explanation of the relation between the translocation of sugars and the translocation of growth regulators has been made. Two possible explanations of this relationship seem worth mentioning. The sugar may act in the cells of the phloem only as a metabolite which provides the energy necessary for the translocation of the growth regulator. Or some reaction such as esterification may occur between the sugar and the growth regulator and the growth regulator may be translocated only when combined in such a product. Perhaps such a reaction may take place in the plant only through phosphorylation.

If either of these hypotheses is correct, then the relation between phosphorus nutrition and the translocation of 2,4-D would be straightforward. In the first case, phosphorus would be necessary for the utilization of the sugar as an energy source; in the second case it would be necessary for the formation of the translocatable material.

It should be pointed out that while the tomato plants in these experiments were suffering from extreme phosphorus deficiency when they were treated, most of them recovered when phosphorus was added. Phosphorus deficiency has been shown to result in an increase in the sugar content of tomato plants (1, 6, 8) and therefore the low rate of translocation of the 2,4-D must not have resulted from a low sugar content.

Summary

Phosphorus deficient tomato plants which were supplied with phosphorus 3 days before the application of 2,4-D labeled with C^{14} had significantly greater stem curvatures, significantly lower fresh weight of tops, and much more radioactive material in the hypocotyls and roots than did the plants which did not receive phosphorus until 24 hours after treatment with the growth regulator. Plants which received phosphorus 8 hours before or at the time of treatment with 2,4-D were intermediate in their growth responses and in the amount of radioactive material in the hypocotyls and roots.

It appears that 2,4-D is not readily translocated through tomato plants which are seriously deficient in phosphorus.

Literature Cited

1. Eckerson, S. H. Influence of phosphorus deficiency on metabolism of tomato (*Lycopersicum esculentum* Mill.). Contrib. Boyce Thompson Inst. 3: 197–217. 1931.
2. Ennis, W. B., Jr. and Boyd, F. T. The response of kidney-bean and soybean to aqueous-spray applications of 2,4-dichlorophenoxyacetic acid with and without Carbowax. Bot. Gaz. 107: 552–559. 1946.
3. Gauch, H. G. and Dugger, W. M., Jr. The role of boron in the translocation of sucrose. Plant Physiol. 28: 457–467. 1953.
4. Holley, R. W., Boyle, F. P. and Hand, D. B. Studies on the fate of radioactive 2,4-dichlorophenoxyacetic acid in bean plants. Arch. Biochem. 27: 143–151. 1950.
5. Jaworski, E. G. and Butts, J. S. Studies in plant metabolism. II. The metabolism of C^{14}-labeled 2,4-dichlorophenoxyacetic acid in bean plants. Arch. Biochem. Biophys. 38: 207–218. 1952.
6. Kraybill, H. R. Plant metabolism studies as an aid in determining fertilizer requirements. I. Ind. Eng. Chem. 22: 275–276. 1930.
7. Linder, P. J., Brown, J. W. and Mitchell, J. W. Movement of externally applied phenoxy compounds in bean plants in relation to conditions favoring carbohydrate translocation. Bot. Gaz. 110: 628–632. 1949.
8. MacGillivray, J. H. Effect of phosphorus on the composition of the tomato plant. Jour. Agr. Research 34: 97–127. 1927.
9. Mitchell, J. W. and Brown, J. W. Movement of 2,4-dichlorophenoxyacetic acid stimulus and its relation to the translocation of organic food materials in plants. Bot. Gaz. 107: 393–407. 1946.
10. Mitchell, J. M., Dugger, W. M., Jr. and Gauch, H. G. Increased translocation of plant-growth-modifying substances due to application of boron. Science 118: 354–355. 1953.
11. Rice, E. L. Absorption and translocation of ammonium 2,4-dichlorophenoxyacetate by bean plants. Bot. Gaz. 109: 301–314. 1948.
12. Rohrbaugh, L. M. and Rice, E. L. Effect of application of sugar on the translocation of sodium 2,4-dichlorophenoxyacetate by bean plants in the dark. Bot. Gaz. 111: 85–89. 1949.
13. Weaver, R. J. and DeRose, H. R. Absorption and translocation of 2,4-dichlorophenoxyacetic acid. Bot. Gaz. 107: 509–521. 1946.
14. Weintraub, R. L. and Brown, J. W. Translocation of exogenous growth-regulators in the bean seedling. Plant Physiol. 25: 140–149. 1950.
15. Weintraub, R. L., Yeatman, J. N., Lockhart, J. A., Reinhart, J. H. and Fields, Melvin Metabolism of 2,4-dichlorophenoxyacetic acid. II. Metabolism of the side chain by bean plants. Arch. Biochem. Biophys. 40: 277–285. 1952.

THE RELATION BETWEEN IRON AND CHLOROPHYLL CONTENTS IN CHLOROTIC SUNFLOWER LEAVES[1]

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The advent of highly stable iron complexes which are useful in plant nutrition and agriculture (9, 16) has led to a renewed interest in the study of iron nutrition, particularly in lime-induced chlorosis. However, the investigator soon faces the problem of the comparative iron status of green and chlorotic leaves. Some investigators have found less iron in chlorotic than in green leaves and have demonstrated a good correlation between iron and chlorophyll contents (8, 15, 18). But also, it has been found that chlorotic leaves may contain as much or more iron than comparable green leaves (7, 11, 19). Brown and Hendricks (2) state that the concentration of iron in plant tissues has not been a satisfactory index of deficiency. Consequently chlorosis is frequently considered to involve more than a simple iron deficiency. Leeper (10) has summed up this view in the following statement with reference to lime-induced chlorosis. "Either the iron enters the plant in a less useful form than normal, or something goes wrong with the reactions of iron inside the plant growing on certain soils. The latter seems more likely."

Although Iljin (7) did not believe lime-induced chlorosis to be due simply to a deficiency of iron, he and others have pointed out that many if not all of the observed changes in the composition of chlorotic plants are consequences and not causes of chlorosis. De Kock and Hall (4) have examined the chlorosis occurring in variegated plants and that caused by virological or pathological causes and found the same high phosphorus-iron and low calcium-potassium ratios as is found in other forms of chlorosis. Probably the only major reason that simple iron deficiency has not been generally accepted as the cause of chlorosis is due to the continuing reports that as much or more iron may be present in chlorotic leaves as in comparable green leaves.

Some time ago, it was pointed out that surface contamination of leaves with iron might account for the reported lack of correlation between iron and chlorophyll contents (8) and it was suggested that adequate washing of leaves was of great importance in obtaining positive correlations. Since then, thorough washing has become more or less a general procedure but conflicting results are still being reported. Thus it would appear necessary to establish definitely whether iron chlorosis is caused directly by lack of iron or by more complex phenomena involving metabolic disarrangements.

In a study of the absorption and translocation of

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ethylenediamine tetraacetic acid, Weinstein et al (19) reported that sunflowers grown at pH 7 with $FeSO_4$ as the source of iron gave rise to chlorotic leaves containing as much total iron as green leaves from plants grown under similar conditions at pH 5. This appeared to provide a means of readily obtaining chlorotic leaves with iron contents as high as in comparable green leaves. Experiments were therefore undertaken to see if a stock of material containing "non-utilizable" iron could be obtained.

Materials and Methods

Initial experiments were carried out closely following the procedure of Weinstein et al. In these experiments, sunflower (*Helianthus annuus* L.) was the test plant. The seeds were germinated in sand and seven days later the seedlings were transplanted into aerated nutrient solutions. The plants were harvested about 4 weeks after transplanting. The composition of the nutrient solutions used for the first two experiments was just as described by Weinstein et al. Hoagland nutrient solution (0.005 M KNO_3, 0.005 M $Ca(NO_3)_2$, 0.002 M $MgSO_4$, 0.001 M KH_2PO_4, 0.5 ppm Mn, 0.5 ppm B, 0.05 ppm Zn, 0.02 ppm Cu and 0.01 ppm Mo) was used in the later experiments. Reagent grade chemicals were used to prepare stock solutions for the first experiment. In subsequent experiments, chemicals especially purified by the method of Stout and Arnon (17) were used. Distilled water was used for all nutrient solutions. In addition to the usual micronutrients, 3 ppm Cl as KCl was added since the recent work of Broyer et al (3) indicates that this element is probably essential. Surfaces in contact with the nutrient solution were painted with asphaltum varnish or Amercoat paint (Amercoat Corporation).

After harvesting, the fresh material was separated into various plant parts and each fraction weighed. The leaf samples which consisted of several leaves of similar age and appearance were carefully washed in a 2 % solution of Dreft detergent and rinsed with distilled water. The washed samples were dried at 65° C in an oven with forced ventilation. After drying, the material was weighed and ground using a glass mortar and pestle. Contact with iron or iron containing substances was avoided at all times. In those cases where chlorophyll was to be determined, samples were removed prior to the washing and the chlorophyll determined within a short period of time.

Total iron was determined colorimetrically by the ortho-phenanthroline method after ashing a suitable amount of the ground material with redistilled concentrated H_2SO_4 and iron free 30 % H_2O_2 (8).

Samples (about 2 gm) for chlorophyll determination, obtained from the fresh unwashed material were weighed and ground with washed sand using a pinch of $CaCO_3$ and a few ml of acetone. The chlorophyll was extracted with 80 % (v/v) acetone-water and the absorption of the resulting solution measured at 663 and 645 mμ with a Beckman Model B spectrophotometer. Concentrations were calculated from this data and the values for the specific absorption coefficients

Table I

Iron Contents of Leaves from Sunflower Plants Grown at pH 5 and pH 7. Solutions Changed Every 2nd Day and 0.5 ppm Iron Supplied as $FeSO_4$

pH	Plant no.	Ppm total Fe			Description
		Young	Recently matured	Old	
5	1	51.9	61.0	72.3	Very slightly chlorotic
	2	62.8	65.3	75.8	" " "
	3	68.8	66.3	70.4	" " "
5	1	70.4	78.6	78.4	Very slightly chlorotic
	2	57.8	59.5	68.1	Slight chlorosis
	3	81.0	74.2	73.1	Trace of chlorosis
5	1	40.3	44.7	60.3	Slight chlorosis
	2	49.8	46.8	58.9	Young leaves slightly chlorotic, old leaves green
	3	49.6	50.7	61.2	" " " " " " "
7	1	26.8	27.9	29.9	Moderate chlorosis, veins green
	2	26.3	26.0	33.7	Moderate chlorosis
	3	29.2	31.8	40.3	Moderate chlorosis, veins green
7	1	24.3	32.3	33.0	Moderate chlorosis
	2	29.4	32.7	33.5	" "
	3	24.5	33.6	36.3	" "
7	1	20.9	23.6	27.1	Severe chlorosis, some green veins
	2	27.1	28.4	37.2	" " " " "
	3	37.7	41.5	52.0	Slight to moderate chlorosis

Av. dry wt of entire plant = 14.5 gm for pH 5 set and 8.9 gm for pH 7 set.
Av. Fe content of stems = 15 ppm for pH 5 set and 7 ppm for pH 7 set.
Av. Fe content of petioles = 13 ppm for pH 5 set and 8 ppm for pH 7 set.
Av. Fe content of roots = 3000 ppm for pH 5 set and 1200 ppm for pH 7 set.

of chlorophyll a and b published by Mackinney (12). All data in the tables are expressed on a dry weight basis.

Results

Constant Iron Supply: For the first experiment, three 15-l crocks containing 3 sunflower plants each were maintained at pH 5 and 3 similar crocks were maintained at pH 7. The solutions were renewed every other day. 0.5 ppm Fe as $FeSO_4$ was added each time the solutions were renewed. An attempt was made to keep the pH constant by frequent adjustment between renewals. The seedlings were transplanted into the solutions on August 22 and the plants harvested 26 days later. The harvested leaves were classified according to age and degree of chlorosis. The data are given in table I.

Even the plants grown at pH 5 were not completely green although nearly so. Evidently climatic and cultural conditions were such that 0.5 ppm Fe was not quite sufficient. Nevertheless, the data of table I clearly show a correlation between iron content and degree of chlorosis. As a group, the leaves from plants grown at pH 5 were only slightly chlorotic and contained approximately 40 to 80 ppm Fe. The leaves of plants grown at pH 7 were moderately to severely chlorotic and contained about 20 to 40 ppm Fe. The only exception was the sample of old leaves from plant no. 3 listed at the bottom of the table. These leaves contained 52 ppm Fe but these plants were the least chlorotic of the pH 7 group. Analyses of other plant parts gave a similar picture. The iron values for the roots are probably not very meaningful because of the difficulty of removing precipitated iron from the root surface.

The results of this experiment differed so drastically from those reported by Weinstein et al (19) that a similar experiment was performed. This second experiment was carried out just as the first experiment except especially purified reagents were used in making up stock solutions. Rather than classify leaves according to appearance, the chlorophyll contents were determined. The seedlings were transplanted March 8 and the plants harvested 24 days later. The data of this experiment are summarized in table II. At this time of the year, better growth was obtained than in the previous experiment. The yields were considerably higher and not too dissimilar from those obtained by Weinstein et al (19).

As can be seen from the data of table II, there is a very good correlation between the amounts of total iron and chlorophyll with the exception of one sample of old leaves.

A comparison of the results of the above two experiments with those of Weinstein et al (19) emphasizes the problem previously stated; namely, some investigators find a correlation between iron and chlorophyll contents and others find no correlation whatever.

Fluctuating Iron Supply: In the following experiments, the iron-chlorophyll relationships were examined as a function of the variability of the rate of iron supply to the plant root. On April 8, sunflower seedlings were transplanted to complete Hoagland nutrient solution containing 0.1 ppm Fe as ferric sodium ethylenediamine tetraacetate (Fe EDTA). Subsequently on April 21, the plants were transferred to 15-l crocks, 3 plants to a crock. The Hoagland nutrient solution used in these crocks was prepared from especially purified stock solutions and no iron was added. These solutions were not changed during the remainder of the experiment. By May 6, chlorotic symptoms were well developed. On this date, leaf samples were taken for chlorophyll and iron analyses from one group of plants and 10 ppm Fe as Fe EDTA were added to the nutrient solution. One week later, leaf samples were again taken from this treated group of plants. Another group of plants was permitted to remain in the iron free solution until May 11, initial samples taken and 10 ppm Fe as Fe EDTA added. Samples were again taken one week later.

The data for these experiments are presented in table III. The left side of the table gives the chlorophyll and iron data before treatment with iron and the right side gives the data one week after the addition of iron to the nutrient solution. The samples are arranged on the basis of chlorophyll content, i.e., the first sample on the right side of the table did not necessarily come from the same plant which furnished the first sample on the left side. In this experiment, both the initial and final samples consisted of matured and recently matured leaves.

An examination of the left side of the table shows that a reasonably good correlation between chlorophyll and iron contents was obtained by omitting iron from the nutrient solution. A comparison of this data with that from table II indicates that growth

Table II

Iron and Chlorophyll Contents of Leaves from Sunflower Plants Grown at pH 5 and pH 7. Solutions Changed Every 2nd Day and 0.5 ppm Iron Supplied as $FeSO_4$

pH	Age of leaf	Ppm Fe	% Chlorophyll
5	R.m.*	88.2	0.827
	Old	101.0	0.678
5	R.m.	84.9	0.684
	Old	94.5	0.793
7	R.m.	55.5	0.538
	Old	59.0	0.570
7	R.m.	52.8	0.406
	Old	51.2	0.503
7	R.m.	40.6	0.288
	Old	38.7	0.377
7	R.m.	35.2	0.241
	Old	41.1	0.272

* R.m. = Recently matured.

Av. dry wt of entire plant = 24.0 gm for pH 5 set and 16.4 gm for pH 7 set.

TABLE III

THE EFFECT OF A FIFTEEN AND TWENTY DAY INTERRUPTION OF THE IRON SUPPLY ON THE IRON AND CHLOROPHYLL CONTENTS OF MATURED AND RECENTLY MATURED SUNFLOWER LEAVES. IRON SUPPLY RESUMED AT 10 PPM LEVEL WITH Fe EDTA

INTERRUPTION OF IRON SUPPLY	BEFORE ADDITION OF Fe		ONE WEEK AFTER ADDITION OF Fe	
	PPM Fe	% CHLOROPHYLL	PPM Fe	% CHLOROPHYLL
15-Day	14.8	0.0088	835	0.060
	19.5	0.0835	389	0.185
	21.4	0.165	490	0.192
	27.8	0.343	258	0.412
	27.8	0.432	442	0.458
	37.7	0.585	267	0.849
20-Day	18.1	0.0381	297	0.214
	22.6	0.152	543	0.450
	25.4	0.204	318	0.458
	24.2	0.252	525	0.651
	24.3	0.283	442	0.744
	34.4	0.486	359	0.722 *
	...	....	462	0.651 **
	...	....	462	0.286 †

* Green basal portions of leaf sample.
** Midsections of above sample.
† Chlorotic apical sections of above sample.

conditions in this experiment were such that iron was utilized more efficiently, the chlorophyll to iron ratio being considerably higher. It is quite apparent that the addition of iron to the previously iron free nutrient solution completely destroys the correlation between chlorophyll and iron. Furthermore, abnormally high iron contents are found in the leaves of plants so treated. When several leaves which were green at the base and chlorotic at the apex were examined, the same lack of correlation and extremely high iron content existed over the whole leaf. Although the entire leaf accumulated considerable iron, only a portion of the leaf responded by increased chlorophyll production.

In another experiment, the iron supply to the roots was interrupted for varying periods and the response observed. Sunflower seedlings were transplanted into Hoagland solution containing 1 ppm Fe as Fe EDTA on July 7. On July 18, the plants were transferred to nineteen 15-l crocks containing purified Hoagland solution but no iron. Each crock held 5 plants. One ppm Fe as Fe EDTA was added on the same day to the first crock. On the next day, 1 ppm Fe was added to the second crock. Addition of iron was made to individual crocks at subsequent intervals as listed in table IV. In this way the iron supply was interrupted for periods of time ranging from 0 to 30 days. Since the chlorotic plants made relatively little growth, it was neither necessary nor desirable to change the solutions. During this period a small amount of iron contamination kept the plants growing although at a reduced rate and they became progressively more chlorotic.

Initial samples for iron and chlorophyll analyses were taken from the plants in each crock at the conclusion of the minus iron treatments just before the addition of iron. Final samples were taken one week after iron had been added to the solution. In table IV, values for iron and chlorophyll contents are given for the initial and final samples. The initial samples consisted of recently matured leaves and the final samples consisted of recently matured and of old leaves.

In a general way the higher the iron content, the higher the chlorophyll content of the initial samples. A closer correlation was not obtained possibly because of the erratic nature of the small amount of iron contamination present and the long time interval between taking the first and last samples. Even in a most general way, there was no relation between the iron and chlorophyll contents in the final samples. The recently matured leaves were all high in chlorophyll content and contained widely differing amounts of iron. The older leaves which would correspond to the recently matured leaves of the initial samples also contained varying amounts of iron. These older leaf samples had lesser amounts of chlorophyll than the final recently matured leaves indicating that they had not completely recovered from chlorosis. The abnormally high iron values, previously observed, appeared possibly after a 2-day interruption and certainly after a 3-day interruption of the iron supply. The iron contents reached a peak in those plants subjected to a 12- to 14-day minus iron treatment.

TABLE IV

THE EFFECT OF VARYING THE DURATION OF THE INTERRUPTION OF THE IRON SUPPLY ON THE IRON AND CHLOROPHYLL CONTENTS OF SUNFLOWER LEAVES. IRON SUPPLY RESUMED AT 1.0 PPM LEVEL WITH Fe EDTA

PRETREATMENT—DAYS IN −Fe SOLUTION	BEFORE ADDITION OF Fe RECENTLY MATURED LEAVES		ONE WEEK AFTER ADDITION OF Fe			
			RECENTLY MATURED LEAVES		OLD LEAVES	
	PPM Fe	% CHLOROPHYLL	PPM Fe	% CHLOROPHYLL	PPM Fe	% CHLOROPHYLL
0	56.9	0.792	77.0	0.855	...	....
1	60.3	0.696	77.6	1.00	...	....
2	64.5	1.00	93.8	0.893	...	....
3	49.0	0.704	132	1.03	...	....
4	45.1	0.648	294	1.03	582	0.891
5	55.9	0.740	240	1.10	...	....
6	44.2	0.660	214	1.07	381	0.969
8	34.0	0.523	259	0.797	344	0.571
10	33.7	0.593	265	0.986	247	0.670
12	42.0	0.761	544	1.01	639	0.507
14	35.8	0.587	620	1.13	482	0.757
16	27.8	0.355	227	1.00	234	0.702
18	30.9	0.486	198	1.16	431	0.516
20	45.0	0.573	140	1.12	136	0.642
22	32.5	0.475	332	0.975	344	0.580
24	23.6	0.357	265	1.13	227	0.389
26	25.3	0.260	299	0.858	223	0.678
28	38.0	0.661	288	0.812	201	0.576
30	31.3	0.438	250	1.05	151	0.604

Four weeks after the end of the minus iron treatment, the iron and chlorophyll contents of recently matured leaves from plants whose iron supply was interrupted for 2, 3 and 4 days were examined. The chlorophyll content of these leaves which had developed some time after the iron supply was resumed varied from 0.799 to 1.28 % and the iron content varied from 44.7 to 73.5 ppm. By this time, an essentially normal relationship between iron and chlorophyll contents had been reestablished.

In the above experiments, the accumulation of iron and the irreversibility of chlorosis occurred in plants growing in solutions in which Fe EDTA was used as the source of iron. A check experiment using $FeSO_4$ gave very similar results, indicating that these effects are related to iron per se and are not peculiar to the complex.

Discussion

A lack of relationship between iron and chlorophyll contents was demonstrated by Weinstein et al (19) using greenhouse grown sunflowers. However, in our hands, similar experiments gave rise to plants showing a good correlation between iron and chlorophyll contents. Evidently some factor of which neither we nor they were cognizant was involved.

Even though nutrient solutions were changed every other day in the first two experiments described here, quite large and rapid changes in the pH of the nutrient solutions were observed. The changes were caused by selective absorption of ions by the roots and were related to general growth factors. Changes in pH affect the availability of iron when supplied as $FeSO_4$. At high pH, rapid oxidation and precipitation occurs. At low pH, iron is brought into solution. This behavior would be particularly prominent in the pH 7 cultures. In our experiments, an attempt was made to minimize the pH changes by frequent adjustment between renewals of the nutrient solution. Even with adjustment, pH decreases of a unit or more were occasionally observed in the pH 7 cultures. Since Weinstein et al (19) apparently did not control pH between renewals, their pH 7 plants might well have been exposed to a fluctuating iron supply. This could produce results comparable to those obtained by deliberately interrupting the iron supply.

The data of tables I, II and the left side of table III are typical of the results consistently found in this laboratory in the study of iron nutrition. For any given set of plants, a reasonably smooth curve is obtained by plotting the chlorophyll content against the iron content (8). The efficiency of the iron, i.e., the slope of the curve, varies from one set to another and is influenced by climatic and other factors.

The above relationship has been observed when the rate of iron supply was uniform or nearly uniform. If the rate is interrupted drastically, then very different results are obtained. After the iron supply is resumed, it is found that the correlation between iron and chlorophyll no longer exists and abnormally large amounts of iron are present in the leaves. The large increase in iron content occurs in old leaves and in leaves developed immediately after the resumption of the iron supply. Later growth contains considerably smaller amounts of iron.

The increased iron content resulting from a temporary interruption of the iron supply is not confined to chlorotic tissue only. It is found in the green leaves and green parts of semi-chlorotic leaves. Analytical data and autoradiographs with radioiron show no detectable differences in the amount of accumulation in green and chlorotic tissues. Presumably, the roots temporarily acquire a high capacity for the absorption of iron after being subjected to a minus iron treatment. This behavior is somewhat comparable to the enhanced uptake of salts exhibited by excised low salt barley roots. The high iron content of the leaf is similar to the "luxury consumption" of nutrients observed in foliar diagnosis.

In addition to the increased iron accumulation another equally important factor which may destroy the correlation between iron and chlorophyll contents is the observation that chlorosis tends to be irreversible except possibly in very young leaves. Slight or mild chlorosis shows good recovery on the addition of iron but more severely chlorotic leaves are incapable of turning green or at best do so only partially (13). When plants having chlorotic leaves were placed in solutions containing iron, the iron content of the leaves increased considerably but the yellow leaves never became completely green again except in cases of very mild chlorosis.

The inability of severely chlorotic leaves to recover completely may best be explained on the basis that iron is essential, directly or indirectly, for the production of chlorophyll and a deficiency of iron irreversibly damages the producing mechanism. Iron deficiency has been reported to change the relative activities of some plant enzymes (2). In green leaves, a considerably larger proportion of the nitrogen is present as protein nitrogen than in the case of chlorotic leaves (6), due perhaps to a direct relation between iron and protein synthesis or to a decrease in protein synthesis brought about by a reduced rate of photosynthesis in chlorotic leaves. Good correlations have been found between iron and protein contents as well as between iron and chlorophyll contents (1). Since one-third to one-half of the leaf protein is contained in the chloroplasts (14), large differences in protein content should involve the chloroplasts. When green and chlorotic sugar beet leaves were compared, fewer and smaller chloroplasts were present in the cells of the chlorotic leaf. If replacement of the disintegrated chloroplasts does not occur or occurs to a limited extent, then the leaf would be unable to recover completely from chlorosis. It is interesting that nearly a hundred years ago, Gris concluded that chlorosis was characterized by an imperfect development of the chloroplast caused by lack of iron salts (5).

A deficiency of iron may therefore progressively impair the chlorophyll producing mechanism to the point that chlorophyll synthesis is limited not by the

amount of iron in the leaf but by the inability of the leaf to produce chlorophyll. According to this view, it is not the iron which becomes inactive but rather the leaf becomes inactive because of the preceding period of iron shortage. In sunflower, this point is readily reached, a few days of minus iron treatment sufficing. The chlorotic leaves of other plants may retain some ability to turn green for periods as long as two or three months. An indication of this was observed with chlorotic pear and peach leaves in the field.

Basically, the type of chlorosis studied here is initially caused by a simple deficiency of iron. The deficiency and resulting chlorosis modify the behavior of the plant in such way as to destroy the correlation between iron and chlorophyll contents provided the appropriate conditions are present. In sunflowers, chlorosis is characterized by an increased ability to accumulate iron in the leaves and probably the entire plant and by incomplete reversibility of the chlorosis. When the rate of iron supply fluctuates, these factors appear to be instrumental in obscuring the relation between iron and chlorophyll contents. With a reasonably uniform rate of iron supply, a good correlation is maintained. Although this work does not deal with lime induced chlorosis, it seems highly probable that the above statements may apply equally well in that case.

Summary

1. If iron is supplied at a uniform rate, then good correlations are obtained between iron and chlorophyll contents in sunflower leaves.

2. If sunflower plants undergo a preliminary period of iron deficiency, then no correlation between iron and chlorophyll contents are found when the iron supply subsequently becomes adequate.

3. A deficiency of iron causes the sunflower leaf to accumulate excessive amounts of iron upon restoration of the iron supply, probably because of an enhanced iron uptake by the low iron plant.

4. Chlorosis tends to be irreversible so chlorotic leaves may not turn completely green even when large amounts of iron enter the leaf. It is suggested that iron is involved in chloroplast formation via protein synthesis, directly or indirectly.

5. Chlorosis is initially caused by a simple deficiency of iron but under some conditions, subsequent correlations between iron and chlorophyll contents may be obscured because of effects related to the deficiency.

Literature Cited

1. Bennett, J. P. Iron in leaves. Soil Sci. 60: 91–105. 1945.
2. Brown, J. C. and Hendricks, S. B. Enzymatic activities as indications of copper and iron deficiencies in plants. Plant Physiol. 27: 651–660. 1952.
3. Broyer, T. C., Carlton, A. B., Johnson, C. M. and Stout, P. R. Chlorine—a micronutrient element for higher plants. Plant Physiol. 29: 526–532. 1954.
4. De Kock, Pierre C. and Hall, Alexander The phosphorus-iron relationship in genetical chlorosis. Plant Physiol. 30: 293–295. 1955.
5. Gris, A. Recherches microscopiques sur la chlorophylle. Annales sci. nat. Bot. Ser. 4, 7: 179–219. 1857.
6. Iljin, W. S. Metabolism of plants affected with lime-induced chlorosis (calciose). I. Nitrogen metabolism. Plant and Soil 3: 239–256. 1951.
7. Iljin, W. S. Metabolism of plants affected with lime-induced chlorosis (calciose). III. Mineral elements. Plant and Soil 4: 11–28. 1952.
8. Jacobson, Louis Iron in the leaves and chloroplasts of some plants in relation to their chlorophyll content. Plant Physiol. 20: 233–245. 1945.
9. Jacobson, Louis Maintenance of iron supply in nutrient solutions by a single addition of ferric potassium ethylenediamine tetra-acetate. Plant Physiol. 26: 411–413. 1951.
10. Leeper, G. W. Factors affecting availability of inorganic nutrients in soils with special reference to micronutrient metals. Ann. Rev. Plant Physiol. 3: 1–16. 1952.
11. Lindner, R. C. and Harley, C. P. Nutrient interrelations in lime-induced chlorosis. Plant Physiol. 19: 420–439. 1944.
12. Mackinney, G. Absorption of light by chlorophyll solutions. Jour. Biol. Chem. 140: 315–322. 1941.
13. Molisch, H. Die Pflanze in ihren Beziehung zum Eisen. Pp. 1–119. G. Fischer, Jena 1892.
14. Rabinowitch, Eugene I. Photosynthesis, Vol. I. Pp. 368–376. Interscience Publishers, Inc., New York 1945.
15. Smith, Paul F., Reuther, Walter and Specht, Alston W. Mineral composition of chlorotic orange leaves and some observations on the relation of sample preparation technique to the interpretation of results. Plant Physiol. 25: 496–506. 1950.
16. Stewart, Ivan and Leonard, C. D. Iron chlorosis—its possible causes and control. Citrus Mag. 14 (10): 22. 1952.
17. Stout, P. R. and Arnon, D. I. Experimental methods for the study of the role of copper, manganese, and zinc in the nutrition of higher plants. Amer. Jour. Bot. 26: 144–149. 1939.
18. Wallihan, Ellis F. Relation of chlorosis to concentration of iron in citrus leaves. Amer. Jour. Bot. 42: 101–104. 1955.
19. Weinstein, L. H., Purvis, E. R., Meiss, A. N. and Uhler, R. L. Absorption and translocation of ethylenediamine tetraacetic acid by sunflower plants. Jour. Agr. Food Chem. 2: 421–424. 1954.

THE REDUCTION AND OXIDATION OF GLUTATHIONE BY PLANT MITOCHONDRIA[1,2]

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The reduction of oxidized glutathione (GSSG) by reduced triphosphopyridine nucleotide[3] (reaction *1*)

$$GSSG + TPNH + H^+ \rightarrow 2\,GSH + TPN^+ \qquad (1)$$

is catalyzed by glutathione reductase (3, 4, 5). In earlier studies this reaction has been coupled with soluble dehydrogenases which utilize TPN as a coenzyme (6). Since tissue fractionation studies have shown that numerous dehydrogenases, including those associated with the oxidation of the Krebs cycle intermediates, are localized within the mitochondrion, it was of interest to determine whether these enzymes might also be coupled with reaction *1*. This paper reports that it is possible to demonstrate the reduction of GSSG by plant mitochondria in the presence of suitable substrates. In addition, the plant particles also catalyzed the oxidation of GSH by molecular O_2 in the presence of catalytic amounts of ascorbic acid.

METHODS AND MATERIALS

The avocado mitochondria were obtained from firm fruit (Fuerte and Nabal varieties) purchased in the local market. The isolation procedure employed was that of Biale and Young (7). The mitochondria obtained in this manner were washed by suspending in approximately 150 ml of 0.5 M sucrose and recentrifuging at 17,000 × g. The particles were then suspended in approximately 5 ml of 0.5 M sucrose to make a suspension containing 1 to 2 mg N per ml. Pea mitochondria were obtained from 6- to 12-day-old etiolated pea seedlings (Alaska variety) or from the epicotyls of such plants. The prechilled plant material was ground with 300 ml of 0.5 M sucrose in a cold mortar with coarse, acid-washed sand. The pH of the homogenate was adjusted to 7.0 during grinding and the particles were isolated and washed in the same manner as for the avocado mitochondria. All manipulations were performed at 0 to 4° C and the mitochondria were used immediately after preparation. The nitrogen content of the mitochondria was determined according to Ma and Zuazaga (8). The GSSG reductase analysis is described elsewhere (4).

The reactions were carried out in the Warburg apparatus at 25° C. In the initial experiments all reagents were added to the main compartment of the flask and the mitochondria were placed in the side arm. Ten minutes were allowed for equilibration before tipping in the mitochondria from the side arm to initiate the reaction. In the anaerobic experiments, the vessels were flushed with nitrogen for 5 minutes before equilibration. At the end of the experimental period (60 to 85 minutes) 0.5 ml of 20 % metaphosphoric acid was added and the precipitate centrifuged down and discarded. The supernatant fraction was then analyzed for GSH according to the method of Fujita and Numata (9). The results obtained are expressed as micromoles of GSSG reduced (micromoles of GSH formed divided by 2).

In the experiments on oxidative phosphorylation the above procedure was modified by adding the mitochondria directly to the reaction mixture and placing radioactive inorganic phosphate ($P^{32}O_4^{\equiv}$) in the side arm. After flushing the anaerobic vessels with nitrogen and allowing all vessels to equilibrate for ten minutes, initial readings were taken. The $P^{32}O_4^{\equiv}$ was tipped in from the side arm at this time. After somewhat shorter experimental periods (30 to 40 minutes) the reactions were stopped with 0.5 ml 35 % trichloroacetic acid. The precipitated proteins were centrifuged off and aliquots of the supernatant fraction were analyzed for GSH and inorganic phosphate (10). The remainder of the supernatant fraction was analyzed for esterified P^{32} according to the method of Lehninger (11).

TPN^+ of 70 % purity was generously provided by Dr. M. A. Mitz of Armour Laboratories. DPN^+, AMP-5 and ATP were purchased from Pabst Laboratories; GSSG and GSH from Schwarz, Inc. The $P^{32}O_4^{\equiv}$ obtained from the Atomic Energy Commission was hydrolyzed in dilute acid before use (12).

RESULTS

The oxidation of Krebs cycle intermediates by mitochondria from avocado fruit was first reported by Millerd et al (13). In subsequent studies, Biale and his associates have extensively investigated the enzymatic properties of mitochondria from avocado fruit at all stages of the ripening process, and in particular have determined the optimum conditions for oxidative phosphorylation by these particles (7, 14, 15). With avocado mitochondria as a source of the necessary enzymes, it is also possible to couple the oxidation of the Krebs cycle acids to the reduction of GSSG when GSSG is substituted for O_2 as the oxidizing agent. Table I shows that the oxidation of citrate, succinate, malate and α-ketoglutarate can be carried out with either oxidant. The first column gives the O_2 uptake observed with the particles in air in the absence of GSSG. The second column shows the

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[2] Supported in part by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council. Preliminary reports on some of the data have been made (1, 2).

[3] The following abbreviations have been used: TPN^+ and TPNH, oxidized and reduced triphosphopyridine nucleotide; DPN^+ and DPNH, oxidized and reduced diphosphopyridine nucleotide; GSSG and GSH, oxidized and reduced glutathione; AMP-5, adenosine-5′-PO_4; ATP, adenosine triphosphate.

TABLE I

REDUCTION OF OXIDIZED GLUTATHIONE BY KREBS CYCLE INTERMEDIATES

CONDITIONS	O_2 UPTAKE MICROATOMS/MG N × HR	GSSG REDUCED MICROMOLES/MG N × HR
No substrate	0.7	0.08
Citrate	4.2	3.15
Succinate	12.1	2.28
Malate	2.6	2.01
α-Ketoglutarate ...	10.1	2.59

Reaction mixtures contained 0.02 M substrate; 1.5×10^{-3} M AMP-5; 10^{-3} M $MgSO_4$; 4.5×10^{-3} M GSSG; 6×10^{-5} M TPN^+; 0.01 M phosphate buffer, pH 7.1; 0.3 M sucrose; and 1× washed avocado particles containing 1.01 mg N. Total volume, 2.2 ml. Center well contained 0.1 ml 20% KOH. Gas phase, air or N_2. Reaction time, 80 min.

amount of GSSG reduced when GSSG was added to the reaction mixture and the reaction was carried out in N_2. Since the reduction of one atom of oxygen to H_2O requires two electrons, this reaction is equivalent to the reduction of one mole of GSSG to GSH. The values given in table I and subsequent tables are therefore directly comparable. Although GSSG was readily reduced, it is apparent that the amount of O_2 consumed was generally greater than the amount of GSSG reduced. The variation in O_2 consumption with the different substrates was also greater than the variation in the amount of GSSG reduced and suggests that the reduction of GSSG may be a rate-limiting reaction.

The reduction of GSSG by citric acid was selected for a more detailed study. In these experiments, GSSG was added to incubation mixtures containing the necessary components for the oxidation of citric acid (AMP-5, Mg^{++}, citrate, and mitochondria) and the reactions were carried out in N_2 and in air. As shown in table II, GSSG was reduced both in N_2 and in air. The reaction in both instances was clearly dependent upon the presence of citrate, TPN^+ and GSSG, and the amount of GSSG reduced was the same in air or in N_2. When either Mg^{++} or AMP-5 were omitted from the reaction mixture (not shown), the amount of GSSG reduced was only slightly decreased. The final column of table II also shows the amount of O_2 taken up in the mixture exposed to air. Here again the reaction was dependent upon the presence of an oxidizable substrate. However, O_2 consumption was not dependent on added pyridine nucleotide to the same extent as was GSSG reduction for there was appreciable O_2 uptake in the absence of added pyridine nucleotides. The addition of TPN^+ and DPN^+ approximately doubled and tripled, respectively, the O_2 uptakes. The plant particles apparently contained enough pyridine nucleotides to support some hydrogen transfer to O_2, but these coenzymes were not available for hydrogen transfer to GSSG as the oxidant.

The effect of pH on O_2 uptake and on the coupled reduction of GSSG by the mitochondria preparations was investigated. With citrate as substrate, there was little reaction at pH 6.0 with either O_2 or GSSG as oxidant. Both reactions increased rapidly at pH 7.0 and then decreased again at pH 7.6. With malate as substrate, the reduction of GSSG again exhibited a sharp optimum at pH 7.0, but the reduction of O_2 remained independent of pH from 6.0 to 7.6. The observed optimum of pH 7.0 for the coupled reactions with O_2 or GSSG presumably reflects the overall effect of pH on the several enzyme systems involved, i.e., aconitase, isocitric dehydrogenase, cytochrome reductase, cytochrome c, cytochrome oxidase, and glutathione reductase.

The action of several metabolic inhibitors was also examined. As was expected, sodium azide and potassium cyanide, at a concentration of 0.001 M, inhibited the O_2 uptake 62 and 88% respectively. However, azide and cyanide did not inhibit the reduction of GSSG, and diethyldithiocarbamate (0.001 M) did not affect either the O_2 uptake or GSSG reduction. Since azide and cyanide, but not diethyldithiocarbamate, are effective inhibitors of cytochrome oxidase (16), this enzyme appears to mediate the reduction of O_2, and it is reasonable to assume that a chain of cytochrome enzymes, similar to that found in animal mitochondria, exists in avocado particles. On the other hand, none of these inhibitors inhibit glutathione reductase (17), the enzyme responsible for linking the reduction of GSSG to the dehydrogenases in these preparations.

There is one interesting difference between the reduction of GSSG in air and the same reaction in N_2. The last line of table II demonstrates that DPN^+ will substitute in part for TPN^+ in the reduction of GSSG in air, but cannot substitute in N_2. In view of the fact that GSSG reductase of higher plants is TPN^+ specific (4), this observation can be explained if the DPN^+ is first phosphorylated to TPN^+ by ATP and if

TABLE II

REDUCTION OF OXIDIZED GLUTATHIONE BY CITRIC ACID

CONDITIONS	GSSG REDUCED MICROMOLES/MG N × HR		O_2 UPTAKE MICROATOMS/MG N × HR
	N_2	AIR	
Complete system ..	2.55	2.56	4.5
Omit citrate	0.43	0.53	1.0
Omit TPN^+	0.29	0.18	2.2
Omit GSSG	...	0.03	4.1
Omit GSSG and TPN^+	0.04	0.01	2.7
Substitute DPN^+ for TPN^+	0.38	1.60	6.5

Complete system contained 0.02 M citrate; 1.5×10^{-3} M AMP-5; 10^{-3} M $MgSO_4$; 4.5×10^{-3} M GSSG; 6×10^{-5} M TPN^+; 0.01 M phosphate buffer, pH 7.1; 0.3 M sucrose; and 1× washed avocado particles containing 1.04 mg N. Total volume 2.2 ml. Center well contained 0.1 ml 20% KOH. Gas phase, air or N_2. Reaction time, 85 min.

the formation of ATP is dependent on aerobic conditions. Data supporting this explanation were obtained by comparing the amount of GSSG reduced in air and in N_2 when various combinations of pyridine nucleotides and adenosine phosphates were employed. Table III shows that DPN^+ together with AMP-5 partially substituted for TPN^+ and AMP-5 in air but not in N_2. When, however, ATP was supplied with DPN^+, reduction of GSSG occurred more readily both in air and in N_2. These results, in addition to indicating the phosphorylation of AMP-5 to ATP only under aerobic conditions, suggest the presence in avocado mitochondria of the enzyme described by Kornberg (18) which catalyzes the phosphorylation of DPN^+ to TPN^+ by ATP. The greater reduction of GSSG which occurred in N_2 as compared to O_2 in experiments 1 and 3 was regularly observed and may be due to the fact that, in the experiments carried out in N_2, there is only one oxidant available, namely GSSG.

THE OXIDATION OF GSH BY PLANT MITOCHONDRIA: In addition to catalyzing the reduction of GSSG in the presence of a suitable hydrogen donor, the mitochondria from avocado carried out the oxidation of GSH by O_2 under appropriate conditions. This oxidation is dependent upon the fact that avocado particles contained ascorbic acid oxidase which catalyzes reaction *2*. In a typical experiment (table IV), the

$$\text{Ascorbic acid} + \tfrac{1}{2}\,O_2 \rightarrow \text{Dehydroascorbic acid} + H_2O \qquad (2)$$

rapid and nearly complete oxidation of 11.4 micromoles of ascorbic acid was observed in 20 minutes in the presence of avocado particles. Appropriate blanks were obtained when the substrate was omitted and when the enzyme was heat inactivated. The latter experiment constitutes the control for any autoxidation of ascorbic acid which might occur due to the presence of metallic ions in the reaction mixture. The oxygen uptake was completely inhibited by 0.001 M diethyldithiocarbamate and demonstrates that the oxidation of ascorbic acid was mediated by a copper-containing enzyme, undoubtedly ascorbic acid oxidase.

TABLE III

SUBSTITUTION OF DPN^+ AND ATP FOR TPN^+ IN THE REDUCTION OF OXIDIZED GLUTATHIONE

EXPT NO.	COMPLETE SYSTEM WITH	GSSG REDUCED MICROMOLES/MG N × HR	
		AIR	N_2
1	TPN^+ and AMP-5	3.70	4.12
2	DPN^+ and AMP-5	1.22	0.61
3	DPN^+ and ATP	1.61	2.96
4	Omit TPN^+ or DPN^+	0.35	0.29

Complete system contained 0.02 M citrate; 1.5×10^{-3} M AMP-5 or ATP; 10^{-3} M $MgSO_4$; 3.3×10^{-3} M GSSG; 4.4×10^{-5} M TPN^+ or DPN^+; 0.008 M phosphate buffer, pH 7.1; 0.5 M sucrose; and 1× washed avocado particles containing 0.83 mg N. Total volume, 3.0 ml. Center well contained 0.1 ml 20% KOH. Gas phase, air or N_2. Reaction time 60 min.

TABLE IV

OXIDATION OF ASCORBIC ACID BY AVOCADO MITOCHONDRIA

CONDITIONS	O_2 UPTAKE μL IN 20 MIN
Complete system	123
Omit ascorbic acid	2
Heat inactivated	3
Diethyldithiocarbamate, 10^{-3} M	12

Complete system contained 3.8×10^{-3} M ascorbic acid; 7.5×10^{-2} M phosphate buffer, pH 7.0; 0.5 M sucrose; and 1× washed particles containing 1.27 mg N. Total volume, 3.0 ml. Center well contained 0.1 ml 20% KOH. Gas phase, air.

Cytochrome oxidase, which is also present in these particles, is inhibited only slightly by this concentration of diethyldithiocarbamate (16).

Although ascorbic acid oxidase will not oxidize GSH directly, dehydroascorbic acid, the oxidation product of ascorbic acid, will rapidly oxidize GSH at pH 7.0 and above according to reaction *3* (19).

$$2\ \text{GSH} + \text{Dehydroascorbic acid} \rightarrow \text{GSSG} + \text{Ascorbic acid} \qquad (3)$$

In addition, however, numerous investigators have shown that many plants contain an enzyme, dehydroascorbic acid reductase, which also catalyzes this reaction (see (2) for review). Since particles from avocado can catalyze the oxidation of ascorbic acid (reaction *2*) and since reaction *3* can occur both enzymatically and non-enzymatically, it was possible that the overall reaction, the oxidation of GSH by O_2 (reaction *4*) could occur in the presence of these particles and catalytic amounts of ascorbic acid. Reaction *4*, together with the coupled reduction of GSSG already discussed, would then represent a path of hydrogen transport from substrate through pyridine nucleotides, GSH and ascorbic acid to O_2 which bypasses the cytochrome system.

$$2\ \text{GSH} + \tfrac{1}{2}\,O_2 \rightarrow \text{GSSG} + H_2O \qquad (4)$$

Experiments testing the coupled oxidation of GSH with ascorbic acid and O_2 are presented in table V in which the results are expressed both as μl of O_2 taken up and micromoles GSH oxidized. In the presence of 1.1 micromoles of ascorbic acid, 17.7 micromoles of GSH were oxidized in 40 minutes. In the absence of ascorbic acid there was little reaction, and appropriate blanks (not shown) were obtained when the mitochondrial preparation was heat inactivated. The coupled oxidation of GSH was inhibited 65% (based on O_2 uptake) by 0.002 M diethyldithiocarbamate due to the action of this inhibitor on ascorbic acid oxidase. On the other hand, 0.001 M iodoacetamide inhibited the coupled oxidation of GSH only 33%. Since iodoacetamide is known to inhibit dehydroascorbic acid reductase (20), these data indicated that a substantial amount (67%) of the reaction observed was probably due to the non-enzymatic oxidation of GSH by dehydroascorbic acid. Because of the rapidity of this

reaction at pH 7.0, it was impossible to obtain evidence for dehydroascorbic acid reductase in the avocado preparations.

The avocado particles were not unique in their ability to catalyze the reduction and oxidation of glutathione. Particles isolated from pea seedlings and from mung beans also carried out the reduction of GSSG (2). In addition, the coupled oxidation of GSH by ascorbic acid and O_2 was readily catalyzed by mitochondria obtained from pea hypocotyls.

EXPERIMENTS ON OXIDATIVE PHOSPHORYLATION: Biale and his associates (7, 14, 15) have demonstrated that avocado mitochondria will support phosphorylation during the oxidation of Krebs cycle intermediates. Since the work reported here has shown that GSSG can substitute for O_2 as an oxidant in these preparations, it seemed important to determine whether inorganic phosphate could also be esterified with GSSG as oxidant. Two different procedures for detecting phosphorylation were employed and numerous experiments were performed in which the experimental conditions were widely varied. However, all attempts to demonstrate oxidative phosphorylation with GSSG as the ultimate oxidant were unsuccessful. The initial experiments measured the disappearance of inorganic phosphate in reaction mixtures containing the necessary components for coupling the oxidation of citrate to the reduction of GSSG. Since the avocado particles contained hexokinase, it was only necessary to add glucose to provide a phosphate acceptor for any ATP which might be synthesized. Fluoride was included to prevent loss by phosphatase action. Duplicate vessels were run in N_2 and in air and the amount of inorganic phosphate esterified in each case was measured. While P : O ratios of approximately 1.0 were observed in air with citrate as the substrate, no significant uptake of inorganic phosphate occurred in N_2 with GSSG as the oxidant (table VI).

Although the oxidation-reduction potential of glutathione is not known with certainty, it is intermediate between that of diphosphopyridine nucleotide and that of cytochrome c (21). On thermodynamic grounds, therefore, the ratio of the number of atoms of phosphorus esterified to the number of moles of GSSG reduced (i.e., the P : GSSG ratio) could be 1.0. This figure is arrived at from a consideration of the oxidation-reduction potentials of the DPNH-DPN couple and the most negative value which appears possible for the GSH-GSSG couple (5). Since the P : O ratio observed in these experiments is only about 1.0, it was clear that a method of detecting phosphorylation was needed which was more sensitive than measuring the decrease in inorganic phosphate. In addition, greater sensitivity was also desirable in view of the fact that more citrate was oxidized with O_2 as oxidant than with GSSG. Accordingly, the method employed by Lehninger and his associates (11) in their early studies on oxidative phosphorylation was also employed. Results obtained by this method, which measures the amount of labeled inorganic phosphate incorporated into the esterified phosphorus fractions as a result of oxidative phosphorylation, are given for the experiment described in table VI. While 37 % of the P^{32} was esterified under aerobic conditions, only an insignificant amount of tracer was incorporated with GSSG as the sole oxidant. The failure to observe esterification could not be attributed to any inhibitory action of GSSG or GSH since these substances were added or were produced in the aerobic experiments.

Similar experiments with $P^{32}O_4^{\equiv}$ were carried out with mitochondria from avocado and peas in order to determine if phosphorylation occurred either during the oxidation of ascorbic acid or during the oxidation

TABLE V

OXIDATION OF REDUCED GLUTATHIONE BY AVOCADO MITOCHONDRIA

CONDITIONS	GSH OXIDIZED MICROMOLES	O_2 UPTAKE μL
GSH + ascorbic acid	17.7	119
GSH alone	0.6	11
GSH, ascorbic acid and 10^{-3} M diethyldithiocarbamate	6.2	43
GSH, ascorbic acid and 10^{-3} M iodoacetamide	14.4	82
Theory for GSH added	19.5	109
Theory for ascorbic acid added	1.1	13

Reaction mixture contained 6.5×10^{-3} M GSH; 4×10^{-4} M ascorbic acid; 0.5 M sucrose; 10^{-2} M $MgSO_4$; 7.5×10^{-3} M phosphate buffer, pH 7.1; and 1 × washed avocado particles containing 0.99 mg N. Total volume, 3.0 ml. Center well contained 0.1 ml 20 % KOH. Gas phase, air.

TABLE VI

OXIDATIVE PHOSPHORYLATION WITH AVOCADO MITOCHONDRIA

CONDITIONS	O_2 UPTAKE MICRO-ATOMS	INORGANIC P UPTAKE MICRO-ATOMS	P : O RATIO	GSSG REDUCED MICRO-MOLES	P^{32} ESTERIFIED %
Aerobic experiments					
1. Complete system	11.8	7.7	0.65	1.98	37.0
2. Omit citrate	1.9	0.9	...	0.51	1.3
3. Omit GSSG	12.1	11.6	0.96	0.14	37.0
Anaerobic experiments					
4. Complete system	...	1.8	...	1.54	1.9
5. Omit citrate	...	0.0	...	0.72	1.0
6. Omit GSSG	...	2.2	...	0.18	2.1

Complete system contained 0.02 M citrate; 1.5×10^{-3} M $MgSO_4$; 3×10^{-4} M AMP-5; 3.3×10^{-3} M GSSG; 4×10^{-5} M TPN^+; 0.01 M glucose; 0.01 M NaF; 0.007 M phosphate buffer, pH 7.4; 0.5 M sucrose; and 1 × washed avocado particles containing 1.02 mg N. Total volume, 3.0 ml. Center well contained 0.1 ml 20 % KOH. Gas phase, air or N_2. Each vessel received 7.7×10^5 cpm of $P^{32}O_4^{\equiv}$. Reaction time, 40 min.

of GSH in the presence of catalytic amounts of ascorbic acid. Again, however, negative results were obtained although control experiments with citrate as substrate and O_2 as oxidant demonstrated that the phosphorylation mechanisms in these preparations were intact and operative.

PARTICULATE GSSG REDUCTASE AND ASCORBIC ACID OXIDASE: The coupled reactions observed with plant mitochondria indicated the presence of GSSG reductase and ascorbic acid oxidase in these particles. While it is easy to demonstrate both enzymes qualitatively in avocado and pea mitochondria, attempts to determine quantitatively the amounts of GSSG reductase in these preparations have not been successful. The assay procedure used depended on coupling glucose-6-phosphate and glucose-6-phosphate dehydrogenase to the reduction of GSSG by the plant particles. Under conditions where reductase is limiting, the amount of GSSG reduced in a given time should be proportional to the amount of reductase employed (4). As shown in table VII, these conditions were not obtained with avocado mitochondria and similar effects were observed with particles from peas. The reaction was not proportional to the amount of particles added (expts. 1 and 2); moreover, when GSSG reductase from wheat germ (expt. 3) was added to the particles, the wheat germ activity was almost completely inhibited (expt. 4). Since the addition of AMP-5 greatly increased the amount of GSH formed (expt. 5) by avocado mitochondria, it seemed probable that the failure of the assay system to function properly was due to the splitting of TPN^+ by nucleotidases present in the mitochondria. Because of these difficulties, it has been possible to estimate only approximately the amounts of GSSG reductase present in the particle. Such approximate analyses indicate that the specific activity of well-washed avocado particles varied from one-fourth to more than one-half of the specific activity of the supernatant which contained the soluble proteins and submicroscopic fractions. Because of the inadequacy of the assay procedure, no attempt was made to conduct a careful study of the cellular distribution of GSSG reductase. However, it should be emphasized that this enzyme was not confined to the particulate fraction in the tissues studied, but was also found in the supernatant fraction containing the soluble proteins.

In an effort to determine whether the GSSG reductase was firmly associated or attached to the plant particles, repeated washings with 0.5 M sucrose were carried out. These manipulations failed to dislodge the enzyme, and instead, soluble compounds containing nitrogen were removed thereby increasing the apparent specific activity of the particles with respect to GSSG reductase.

No attempt was made to determine the relative distribution of ascorbic acid oxidase in the plant tissues used in these experiments. There was abundant oxidase activity in the fraction representing the soluble proteins, and the amount of enzyme in the particles probably constituted only a small fraction of the total activity of the tissue. However, as in the case of GSSG reductase, the particulate ascorbic acid oxidase was not removed by extensive washing, and instead the specific activity appeared to increase with that treatment.

TABLE VII

ANALYSIS OF AVOCADO PARTICLES FOR GSSG REDUCTASE

EXPT NO.	ADDITIONS TO ASSAY MIXTURE	GSH FORMED MICROMOLES
1	0.2 ml Avocado mitochondria	2.85
2	0.4 ml Avocado mitochondria	2.76
3	0.9 mg Wheat germ GSSG reductase	8.10
4	(2) + (3)	2.97
5	As in (2) + 3×10^{-3} M AMP-5	7.74
6	As in (2), omit glucose-6-PO_4	0.15

Assay mixture contained 1.7×10^{-3} M glucose-6-PO_4; 3.3×10^{-3} M GSSG; 9×10^{-6} M TPN; 35 μgm/ml Zwischenferment; 0.05 M Tris buffer, pH 7.4. Avocado mitochondria (2.44 mg N/ml) were once washed. Total volume 3.0 ml. Time of incubation, 1 hr at 25° C.

DISCUSSION

The reduction of GSSG catalyzed by plant mitochondria demonstrates that particulate dehydrogenases which oxidize Krebs cycle intermediates can be coupled with GSSG reductase. The dependency of the reduction of GSSG on TPN^+ is in keeping with the known specificity of GSSG reductase of higher plants for TPN^+ (4). No conclusions can be drawn, however, regarding the pyridine nucleotide specificity of the particulate dehydrogenases which coupled to the reduction of GSSG, since information is unavailable regarding the presence of the enzyme transhydrogenase (22) on plant mitochondria. The presence of transhydrogenase on the plant particles would permit hydrogen transfer to TPN^+ from DPNH formed by the action of DPN-specific dehydrogenases. It is also interesting that the oxidation of succinic acid could be coupled to GSSG reduction since there is no direct indication that pyridine nucleotides function in the oxidation of succinate. (There is evidence that DPNH can be oxidized by fumaric acid in the presence of a heart muscle preparation (32).) It is possible that the reduction of GSSG observed with succinate was due to the combined action of fumarase and malic dehydrogenase on the fumarate formed when succinate was oxidized. However, this is not in agreement with the observation that fumarate is only very slowly oxidized by the plant mitochondria.

The oxidation of GSH in the presence of plant particles and catalytic amounts of ascorbic acid demonstrates that these particulates possess a mechanism for the passage of hydrogen from substrate through pyridine nucleotides, glutathione and ascorbic acid to O_2. Mapson and Goddard (3) have previously shown that soluble extracts of pea meal can carry out the reduction of dehydroascorbic acid. Mapson and Moustafa (23) have recently extended these studies

and have observed the complete transfer system from substrate to O_2 in soluble extracts from pea seedlings. Their results differ from ours in one very important aspect: due to the presence of dehydroascorbic acid reductase in the soluble pea extracts, every step in their reaction sequence is enzyme catalyzed. Mapson and Moustafa have also concluded that the soluble GSSG-ascorbic acid pathway accounts for approximately 25 % of the total oxygen consumption of pea cotyledons.

Our results were unable to provide any evidence that the reduction and oxidation of glutathione by plant mitochondria is physiologically significant. The addition of catalytic amounts of GSH and ascorbic acid to mitochondrial preparations which were actively oxidizing succinic or citric acids by means of cytochrome oxidase did not result in an increased O_2 uptake. The rate of reduction of GSSG by plant particles was less than the rate of O_2 uptake observed in the same preparations. Finally, oxidative phosphorylation could not be observed either during the anaerobic reduction of GSSG or during the oxidation of GSH or ascorbic acid. Although these last observations indicate that particulate GSSG reductase and ascorbic acid oxidase cannot function in phosphorylation processes, the results obtained may have been due to the destruction of labile enzyme systems. These experiments on oxidative phosphorylation were performed with the hope of obtaining some additional information on the sites of phosphorylation in the hydrogen-transport scheme from pyridine nucleotide to O_2. Lehninger (24) has recently discussed this problem and work from his laboratory and from Lardy's (25) has provided considerable information.

The finding of GSSG reductase and ascorbic acid oxidase on plant mitochondria is worthy of comment. The former enzyme has been extracted as a soluble protein from wheat germ (4) and dried yeast (5) and is found in the soluble press juices obtained from fresh plant tissues (3, 6). Rall and Lehninger (5) concluded from tissue fractionation studies on rat liver that GSSG reductase was present mostly in the soluble fraction. In addition, Mapson and Moustafa (23) reported that the majority of GSSG reductase of pea seedlings was soluble. Nevertheless, the latter authors were able to observe the enzyme in mitochondria obtained from pea seedlings. The posibility of obtaining artifacts in the apparent localization of an enzyme within cells is well known (26). In plant tissues, these difficulties may be more pronounced due to the release of vacuolar contents during the homogenization of the tissue (27). Nevertheless, GSSG reductase was observed in significant quantities on mitochondria obtained from avocado fruit and pea seedlings. When these particles were isolated in a manner which maintained their structural integrity, extensive washing did not remove the enzyme but did solubilize other nitrogenous material in the particles.

It has recently been possible to isolate mitochondria from lupine cotyledons which carry out oxidative phosphorylation with P : O ratios approaching those observed in animal tissues (28). Such preparations presumably have maintained their structural integrity to a larger degree than the avocado particles which seldom exhibited P : O ratios above 1.0. However, the lupine mitochondria cannot link the reduction of GSSG to the oxidation of Krebs cycle acids even though the particles contain abundant amounts of GSSG reductase. These results therefore suggest that the hydrogen and electron transfer system can be shunted into GSSG reduction only in particles which have been altered considerably from their original state.

The cellular distribution of ascorbic acid oxidase has been discussed by Goddard and Stafford (29). Although usually considered as a soluble enzyme, there is recent evidence that ascorbic acid oxidase is also associated with cell wall material (30). Moreover, Mapson et al (31) have reported that mitochondria from pea seedlings will catalyze the oxidation of ascorbic acid.

Summary

1. The reduction of oxidized glutathione has been coupled to the oxidation of Krebs cycle intermediates in the presence of plant mitochondria.

2. The oxidation of reduced glutathione by molecular oxygen is catalyzed by plant mitochondria in the presence of catalytic amounts of ascorbic acid. This is due to the presence of ascorbic acid oxidase in the mitochondria.

3. With the demonstration of these reactions, a mechanism exists in plant mitochondria for the transfer of hydrogen from Krebs cycle substrate through pyridine nucleotides, glutathione, and ascorbic acid to O_2. Such a mechanism does not involve the participation of cytochromes.

4. Attempts to demonstrate oxidative phosphorylation during the reduction of oxidized glutathione and during the oxidation of reduced glutathione and ascorbic acid were negative.

The authors wish to express their appreciation to Dr. Jacob B. Biale and his associates for making the details of their studies on avocado mitochondria available before publication.

Literature Cited

1. Conn, E. E. and Young, L. C. T. Reduction of oxidized glutathione by plant particles. Federation Proc. 13: 194. 1954.
2. Vennesland, B. and Conn, E. E. The enzymatic oxidation and reduction of glutathione. In: Glutathione, S. P. Colowick et al, ed. Pp. 105–126. Academic Press, New York 1954.
3. Mapson, L. W. and Goddard, D. R. The reduction of glutathione by plant tissues. Biochem. Jour. 49: 592–601. 1951.
4. Conn, E. E. and Vennesland, B. Glutathione reductase of wheat germ. Jour. Biol. Chem. 192: 17–28. 1951.
5. Rall, T. W. and Lehninger, A. L. Glutathione reductase of animal tissues. Jour. Biol. Chem. 194: 119–130. 1952.

6. ANDERSON, D. G., STAFFORD, HELEN A., CONN, E. E. and VENNESLAND, B. The distribution in higher plants of triphosphopyridine nucleotide-linked enzyme systems capable of reducing glutathione. Plant Physiol. 27: 675–684. 1952.
7. BIALE, J. B. Oxidative phosphorylation by cytoplasmic particles of fruits in relation to the climacteric. Proc. Intern. Congr. Bot. 8th Congr., Paris Sect. 11: 390–391. 1954.
8. MA, T. S. and ZUAZAGA, G. Micro-Kjeldahl determination of nitrogen. Ind. Eng. Chem., Anal. Ed. 14: 280–282. 1942.
9. FUJITA, A. and NUMATA, I. Über die jodometrische Bestimmung des Glutathions in Geweben. Biochem. Zeits. 299: 249–261. 1938.
10. GOMORI, G. A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. Jour. Lab. Clin. Med. 27: 955–960. 1942.
11. LEHNINGER, A. L. Esterification of inorganic phosphate coupled to electron transport between diphosphopyridine nucleotide and oxygen, II. Jour. Biol. Chem. 178: 625–644. 1949.
12. MAZELIS, M. and STUMPF, P. K. Fat metabolism in higher plants. VI. Incorporation of P^{32} into peanut mitochondrial phospholipids. Plant Physiol. 30: 237–243. 1955.
13. MILLERD, A., BONNER, J. and BIALE, J. B. The climacteric rise in fruit respiration as controlled by phosphorylative coupling. Plant Physiol. 28: 521–531. 1953.
14. BIALE, J. B. and YOUNG, R. E. Oxidative phosphorylation in relation to ripening of the avocado fruit. Abstr., Western Sect. Amer. Soc. Plant Physiol. P. 3. Santa Barbara, California, June 16 to 18, 1953; Effects of EDTA on oxidations mediated by avocado mitochondria. Abstr., Western Sect. Amer. Soc. Plant Physiol. P. 4. Pullman, Washington, June 22 to 24, 1954.
15. ABRAMSKY, M. and BIALE, J. B. The pyruvate oxidation system in avocado fruit particles. Plant Physiol. 30: xxviii–xxix. 1955.
16. JAMES, W. O. and GARTON, N. The use of sodium diethyldithiocarbamate as a respiratory inhibitor. Jour. Exptl. Bot. 3: 310–318. 1952.
17. VAN HEYNINGEN, R. and PIRIE, A. Reduction of glutathione coupled with oxidative decarboxylation of malate in cattle lens. Biochem. Jour. 53: 436–444. 1953.
18. KORNBERG, A. Enzymatic synthesis of triphosphopyridine nucleotide. Jour. Biol. Chem. 182: 805–813. 1950.
19. BORSOOK, H., DAVENPORT, H. W., JEFFREYS, C. E. P. and WARNER, R. C. The oxidation of ascorbic acid and its reduction in vitro and in vivo. Jour. Biol. Chem. 117: 237–279. 1937.
20. CROOK, E. M. The system dehydroascorbic acid-glutathione. Biochem. Jour. 35: 226–236. 1941.
21. CALVIN, M. Mercaptans and disulfides: some physics, chemistry and speculation. In: Glutathione, S. P. Colowick et al, ed. Pp. 3–26. Academic Press, New York 1954.
22. KAPLAN, N. O., COLOWICK, S. P. and NEUFELD, E. F. Pyridine nucleotide transhydrogenase. III. Animal tissue transhydrogenases. Jour. Biol. Chem. 205: 1–15. 1953.
23. MAPSON, L. W. and MOUSTAFA, E. M. Ascorbic acid and glutathione as respiratory carriers in the respiration of pea seedlings. Biochem. Jour. 62: 248–259. 1956.
24. LEHNINGER, A. L. Oxidative phosphorylation. Harvey Lectures Ser. 49: 176–215. 1953–54.
25. MALEY, G. F. and LARDY, H. A. Phosphorylation coupled with the oxidation of reduced cytochrome c. Jour. Biol. Chem. 210: 903–909. 1954.
26. SCHNEIDER, W. C. and HOGEBOOM, G. H. Cytochemical studies of mammalian tissues: the isolation of cell components by differential centrifugation: A review. Cancer Research 11: 1–22. 1951.
27. LATIES, G. G. The physical environment and oxidative and phosphorylative capacities of higher plant mitochondria. Plant Physiol. 28: 557–575. 1953.
28. CONN, E. E. and YOUNG, L. C. T. Oxidative phosphorylation in lupine mitochondria. Federation Proc. 14: 195–196. 1955.
29. GODDARD, D. R. and STAFFORD, HELEN A. Localization of enzymes in the cells of higher plants. Ann. Rev. Plant Physiol. 5: 115–132. 1954.
30. NEWCOMB, E. H. Effect of auxin on ascorbic oxidase activity in tobacco pith cells. Proc. Soc. Exptl. Biol. Med. 76: 504–509. 1951.
31. MAPSON, L. W., ISHERWOOD, F. A. and CHEN, Y. T. Biological synthesis of L-ascorbic acid: the conversion of L-galactono-γ-lactone into L-ascorbic acid by plant mitochondria. Biochem. Jour. 56: 21–28. 1954.
32. SLATER, E. C. The components of the dihydrocozymase oxidase system. Biochem. Jour. 46: 484–499. 1950.

PINEAPPLE CHLOROSIS IN RELATION TO IRON AND NITROGEN[1,2]

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Pineapple plants grown in soils high in pH or in water-soluble manganese become chlorotic, because of greatly decreased iron availability. At high pH, the solubility of iron is greatly reduced and the physiological activity of iron is rendered ineffective by high concentrations of manganese (5, 25). In both cases, adequate supplies of iron, as solution sprays of ferrous sulfate, remedy the symptoms of chlorosis (30). Chlorosis also often results from inadequate supplies of nitrogen in the soil and may be remedied by additions of this element.

The paradox of the type of chlorosis that results

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from iron deficiency is that many species of plants (weeds) grown adjacently to chlorotic pineapple plants remain green, suggesting that different plants presumably employ different mechanisms for the extraction or intake of iron from the soil.

This paper is concerned with the growth, metabolism and chemical differences in the tissues of pineapple plants grown for 75 weeks in solution cultures supplied with adequate "Plus Fe" and inadequate "Minus Fe" amounts of iron, as ferrous sulfate.

Materials and Methods

The planting material was crowns, i.e., vegetative proliferations at the fruit apex, from a Cayenne clone and weighed 225 ± 25 gm. They were suspended singly in 4-gallon porcelain crocks containing 17 l of tap water for 5 weeks for rooting. At the end of this period, they were selected for uniformity in the number and length of roots and then transferred to the solution cultures.

Approximately 400 plants were grown in solution cultures, prepared with CP chemicals but without additional purification by recrystallization. These chemicals contained, as contaminants, traces of iron which in the "Minus Fe" cultures maintained plant growth at suboptimal levels. The molal concentrations of the nutrient salts in the solution cultures were: 0.001 KNO_3, 0.001 K_2SO_4, 0.001 $Ca(NO_3)_2 \cdot 4\,H_2O$, 0.001 $MgSO_4 \cdot 7\,H_2O$, 0.00025 NaH_2PO_4, 0.000025 $NaB_4O_7 \cdot 10\,H_2O$, 0.00001 $ZnSO_4 \cdot 6\,H_2O$, 0.000005 $CuSO_4 \cdot 5\,H_2O$ and 0.000001 $(NH_4)MoO_4$. The "Plus Fe" cultures contained, in addition to the above, 0.001 molal $FeSO_4 \cdot 7\,H_2O$. All solution cultures were continuously aerated and changed at 5-week intervals. During the change, 10 plants in each series were weighed to ascertain growth and some were withdrawn for chemical analysis. The number of plants withdrawn was varied in accordance with the amounts of tissue necessary for this operation. For each of the 5- and 10-week growth intervals, 10 plants were withdrawn and 6 for each of the subsequent 13 intervals. All such plants were dismembered into leaves, stems, roots and fruits, which were weighed separately. The leaves were segregated, according to previously reported techniques (22), into old B, mature C, active D, and young E leaves; the distinction was based on morphological and physicochemical differences and chronological age. The leaves of each group were cut crosswise into basal 1, transitional 2, low chlorophyllous 3, medial chlorophyllous 4 and terminal chlorophyllous 5 sections. Also, the stems were cut into basal B, medial M and apical A sections. All of the sections of the leaves and stems were weighed and analyzed separately. The methods for the analysis of the tissues have been reported previously (21, 24). The results are given on the fresh weight basis.

Results

Plant and Fruit Weights: The growth of pineapple plants and fruits (fig 1) conforms to an autocatalytic monomolecular reaction (20, 23). Differences in plant growth as measured by weight, between the "Plus Fe" and "Minus Fe" cultures, were first observed at 20 weeks of growth and every 5-wk interval thereafter, and were approximately 150, 200, 250, 300, 600, 900, and 1250 gm for the 20, 25, 30, 35, 40, 45 and 50 weeks of growth, respectively. Hence, at 55 weeks of growth, the approximate period for floral differentiation, the "Plus Fe" cultures weighed 1250 gm more than the "Minus Fe" cultures. The plants of both cultures varied in percentage of fresh weight throughout the 55 weeks of growth. At the period of floral differentiation, the differences were from 4.4 to 14.2 for the entire plant, 4.5 to 13.0 for the leaves, 5.0 to 18.0 for the stems, and 2.5 to 20.0 for the roots.

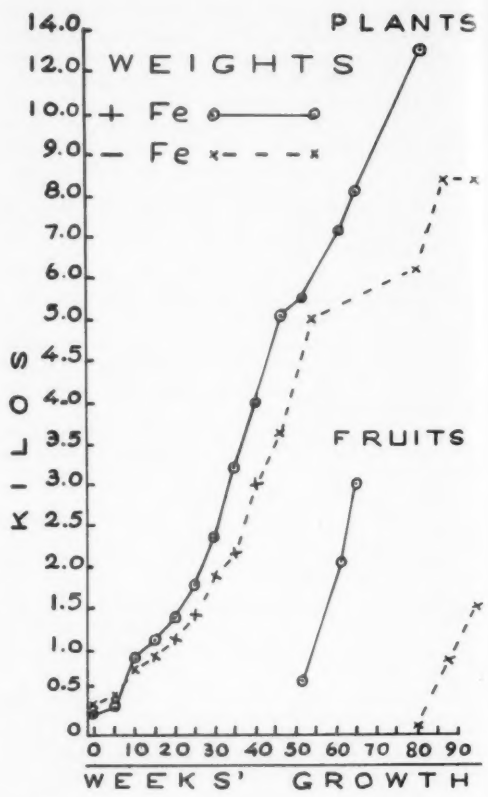


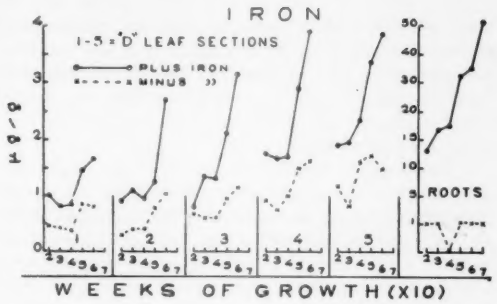
Fig. 1. Combined plant and fruit weights from planting to fruit ripening, also separate fruit weights at 5-wk intervals.

At 55 weeks of growth, approximately 90 % of the plants of the "Plus Fe" and 30 % of the "Minus Fe" cultures differentiated. The remaining 70 % of the plants of the "Minus Fe" cultures differentiated approximately 30 weeks later, suggesting a lower rate of elaboration of substances essential for differentiation than in the "Plus Fe" cultures. Mean fruit weights, at the time of ripening, were approximately twice as great for the "Plus Fe" as for the "Minus Fe" cultures. Also, the quality of the fruit tissues, as deter-

mined by the presence of brown spots, porosity and translucency, was better for the "Plus Fe" than for the "Minus Fe" cultures. The differences in sugar and citric acid content of the fruit tissues between the "Plus Fe" and "Minus Fe" cultures were small and lacked statistical significance.

IRON: Pineapple plants utilize more iron during the period of rapid vegetative growth than before or after. This increased utilization of iron is associated with accelerated chlorophyll synthesis during the rapid expansion of the leaves. With the advent of floral differentiation and cessation of new leaf growth, the iron requirements decrease and gradually increase with the formation of suckers (shoots) and of slips (greatly enlarged crowns with vestigial fruits borne on the peduncle). Chlorophyll first appears in the transitional No. 2 sections of young leaves, where the concentrations of iron usually measure approximately 1.0 μgm/gm fresh weight (fig 2), suggesting that iron is required in relatively small amounts for the activation of chlorophyll synthesis. The comparatively great deposits of iron in the distal sections of the leaves and especially in the terminal No. 5, resulted from translocated iron carried by the stream of transpiration. Usually, the magnitude of iron deposits in the tissues was related to the concentrations of iron in the substratum and to the chronological age of the leaves, the latter being directly associated with the length of the period of iron intake.

The deposits of iron in the roots of the "Plus Fe" series, presumably resulted from greater concentrations of ferrous sulfate in contact with the surface of the roots than the roots could absorb, with the surplus of non-absorbed iron subsequently precipitated as $Fe(OH)_2$ or $Fe_3(PO_4)_2$ in reaction with other substances, in the exodermal tissues. Such iron deposits increased with longer periods of time. Iron did not accumulate in the roots of the "Minus Fe" series, because the intake capacity of the roots for iron exceeded the concentrations in contact with the roots. However, even under iron deficiency, there was a slight tendency for iron accumulation in the distal sections of the leaves carried by the stream of transpiration. Average iron concentrations during the 60 weeks period of plant growth were 1.710 and 0.824 μgm/gm fresh weight for the "Plus Fe" and "Minus Fe" cultures, respectively, that is, 2.1 times as great amounts for the former as for the latter (table I).



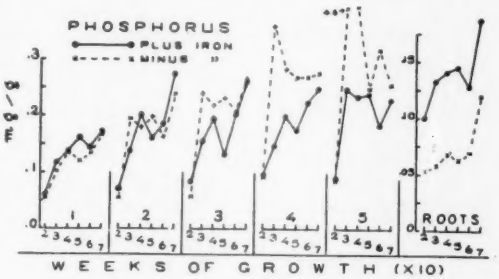


FIG. 2 (*above*). Iron in sections 1, 2, 3, 4 and 5 of D leaves and in the roots at 10-wk intervals.

FIG. 3 (*below*). Phosphorus in sections 1, 2, 3, 4 and 5 of D leaves and in the roots at 10-wk intervals.

Iron accumulated in leaf tissues with increased chronological age, because of longer periods of intake (table II). Average iron concentrations in D leaves of "Plus Fe" cultures were 1.60 and 3.40 μgm/gm, respectively, for 20 and 60 weeks of plant growth. In the "Minus Fe" cultures average iron concentrations

TABLE I

AVERAGE CONCENTRATIONS PER GRAM OF FRESH WEIGHT, FOR 60 WEEKS OF PLANT GROWTH, OF NITRATE, IRON, PHOSPHORUS, POTASSIUM, CALCIUM AND MAGNESIUM IN DIFFERENT SECTIONS OF D LEAVES AND THE RATIOS OF SAME FOR THE "PLUS Fe" AND "MINUS Fe" CULTURES

	NITRATE		IRON		PHOSPHORUS		POTASSIUM		CALCIUM		MAGNESIUM	
	+Fe	−Fe	+Fe	−Fe	+Fe	−Fe	+Fe	−Fe	+Fe	−Fe	+Fe	−Fe
	mg	*mg*	*μgm*	*μgm*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
1	0.472	0.324	1.08	0.59	0.119	0.146	3.53	3.79	0.362	0.324	0.246	0.208
2	0.353	0.226	1.28	0.57	0.175	0.198	4.55	4.97	0.300	0.270	0.196	0.170
3	0.072	0.043	1.63	0.72	0.177	0.223	6.16	6.82	0.424	0.412	0.296	0.262
4	0	0	2.14	1.04	0.185	0.266	7.16	8.14	0.600	0.580	0.446	0.376
5	0	0	2.42	1.20	0.230	0.327	8.07	9.64	0.532	0.532	0.550	0.546
Av.	0.299	0.198	1.710	0.824	0.177	0.232	5.90	6.67	0.444	0.424	0.347	0.312
+Fe/−Fe	1.51		2.08		0.756		0.89		1.05		1.11	

for the 20 and 60 week growth periods were, respectively, 0.76 and 1.32 μgm/gm. Hence, during the interval of 40 weeks, the difference between the two periods, the plants of 60 weeks of growth accumulated iron 2.13 and 1.74 times as great as the ones of 20 weeks of growth for the "Plus Fe" and "Minus Fe" cultures, respectively.

PHOSPHORUS: Phosphorus, as orthophosphate, interferes with the availability of iron to pineapple plants through the formation of insoluble salts. Such salts may form in the substratum at a pH higher than 5.0 or on the exodermal tissues of the roots where both elements tend to accumulate somewhat during the absorption of nutrient solution. The results (fig 3) show that phosphorus translocation from roots to leaves was greatly reduced in the "Plus Fe" cultures by precipitation with iron in the exodermal root tissues. In the "Minus Fe" cultures, with minimal or no precipitation of phosphorus by iron, the concentrations of the former were low in the roots but high in the leaves, a condition diametrically opposite to the one in the "Plus Fe" cultures. Additional proof of reduced availability of phosphorus brought about by iron, or vice versa, appears in the average concentrations of phosphorus in the D leaves for 70 weeks of plant growth in which it was 0.76 times as great in the "Plus Fe" as in the "Minus Fe" cultures (table I). Although the intake of phosphorus was greatly reduced by iron in the "Plus Fe" cultures, the plants developed well, as evidenced by final plant and fruit weights, and without symptoms of phosphorus deficiency.

CHLOROPHYLL: Chlorophyll concentrations, which constitute the most reliable criterion of different degrees of chlorosis, have been employed for the meas-

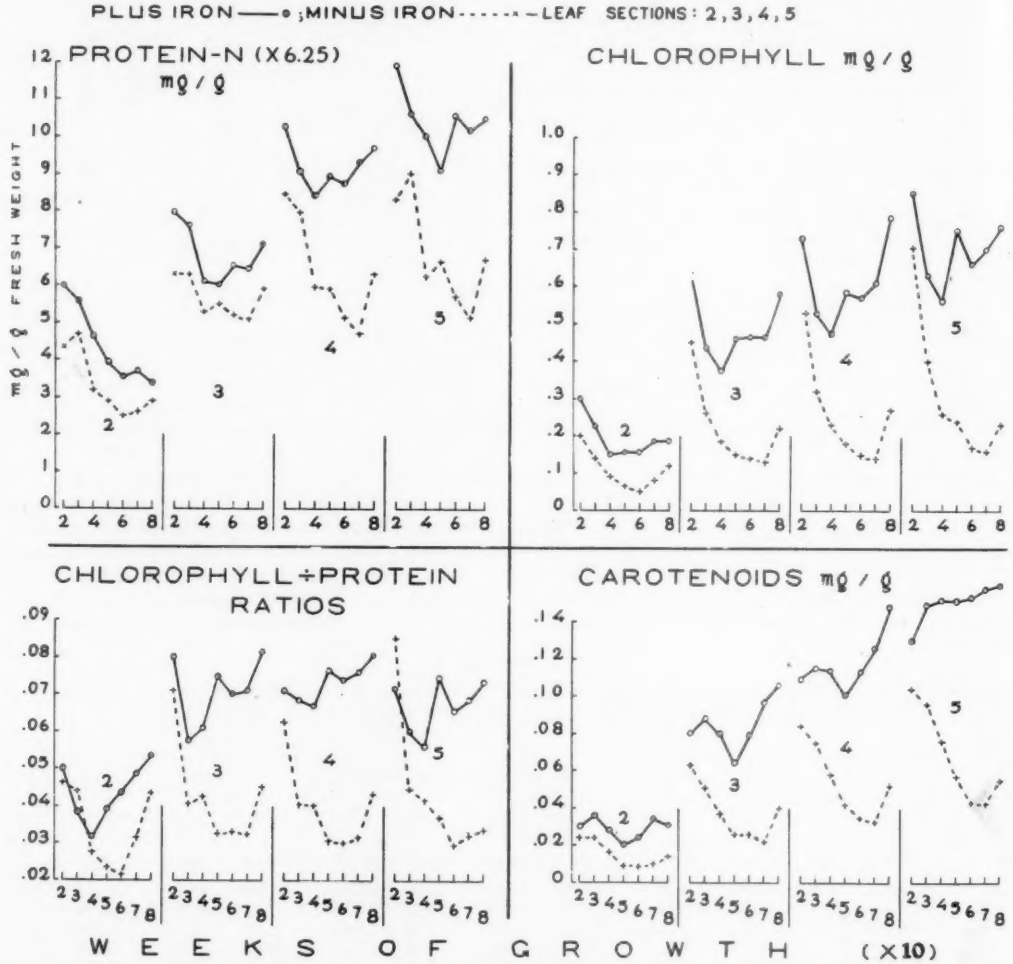

PLUS IRON —•— ; MINUS IRON ------ LEAF SECTIONS: 2, 3, 4, 5
PROTEIN-N (X6.25)
mg / g
mg / g FRESH WEIGHT
CHLOROPHYLL mg / g
CHLOROPHYLL ÷ PROTEIN RATIOS
CAROTENOIDS mg / g
WEEKS OF GROWTH (X10)

FIG. 4. Concentrations of protein (*upper, left*) as protein N × 625, chlorophyll (*upper, right*), carotenoids (*lower, right*), and ratios of chlorophyll to protein (*lower, left*) in sections 2, 3, 4 and 5 of D leaves of "Plus Fe" and "Minus Fe" cultures.

urement of plant performance. In this study, they are compared with iron, carotenoids, protein and plant and fruit weights.

Chlorophyll was extracted from the leaf tissues by the method of Schertz (15) and measured against the standard of Guthrie (4). Chlorophyll and carotenoid concentrations were found to vary greatly in different areas, or sections, along the length of pineapple leaves (20, 22, 26, 27, 28). The measurements reported herein for the "Plus Fe" and "Minus Fe" cultures were made in comparable leaf areas.

The results (fig 4) show that chlorophyll concentrations in the "Plus Fe" cultures declined gradually from the 20th to the 50th week of plant growth. This condition presumably resulted from rapid vegetative growth during which plant growth rates exceeded the rates of chlorophyll synthesis and deposition in the tissues of D leaves. But, after the 50th week of growth with the advent of floral differentiation and cessation of new leaf growth, chlorophyll concentrations greatly increased. However, in the "Minus Fe" cultures chlorophyll concentrations decreased gradually almost until the period of fruit ripening.

Average chlorophyll concentrations in the D leaves for the growth period of 75 weeks (table III) were 0.49 and 0.23 mg respectively, for the "Plus Fe" and "Minus Fe" cultures, that is, 2.12 times as much chlorophyll in the former as in the latter cultures. Chlorophyll synthesis in terms of iron efficacy, as obtained in this experiment, was highly variable, because concentrations of iron greatly increased with greater concentrations in the solution culture and longer periods of growth and of iron intake. For example, in the "Plus Fe" cultures, the data (table II) show that average concentrations of chlorophyll were 1.57 $(0.63 \div 0.40)$ and of iron 0.47 $(1.60 \div 3.40)$ times as great in the 20 as in the 60 weeks of plant growth. In the "Minus Fe" cultures, the concentrations of chlorophyll were 3.54 and of iron 0.58 times as great for the 20 as for the 60 weeks of plant growth. Hence, the amounts of chlorophyll produced per μgm of iron were 0.394 mg and 0.117 mg for the 20 and 60 weeks of plant growth, respectively, in the "Plus Fe" cultures, and in the "Minus Fe" cultures they were, respectively, 0.606 and 0.099 mg. It is quite evident that during the 20 weeks of plant growth, the traces of iron in the "Minus Fe" cultures yielded approximately 1.5 times as much chlorophyll per unit of iron as the greater amounts in the "Plus Fe" cultures. Therefore, the amounts of iron necessary for the activation of chlorophyll synthesis were very small and the accumulations in the plants of 60 weeks growth in the "Plus Fe" cultures should be attributed to iron that is not physiologically active, because it was carried to distal leaf sections by the transpiration stream.

The efficacy of iron for increasing the concentrations of chlorophyll in plants with adequate nitrogen, depends on a synchronization of the rates of iron intake with plant growth and chlorophyll synthesis, as stated earlier (18).

With respect to the chlorophyll concentrations of the "Minus Fe" cultures, occasional analyses of the solution cultures at weekly intervals, disclosed that they were depleted of all traces of iron at the end of the first week after the renewal of the solutions at the regular 5-wk period. Such findings suggested that chlorophyll synthesis was greatly, if not completely, arrested after the depletion of iron. Since this condition was repeated at 5-wk intervals, chlorophyll concentrations declined gradually with advancing plant age.

Carotenoids: The carotenoids, extracted after the chlorophyll (15) from pineapple leaves and comprising both carotene and xanthophyll, occur in an approximate ratio of 1 part of carotenoids to 5 parts of chlorophyll. The results indicate (fig 4) that throughout the entire period of plant growth the concentrations of carotenoids and chlorophylls follow similar patterns. Average carotenoid concentrations of D leaves for 75 weeks of plant growth were 0.094 and 0.046 mg/gm for the "Plus Fe" and "Minus Fe" cultures, respectively. Hence, carotenoid concentrations were 2.04 times as great in the "Plus Fe" as in the "Minus Fe" cultures and this ratio compared well with 2.13, the ratio of chlorophyll concentrations. Chlorophyll and carotenoids correlated in the "Plus Fe" and "Minus Fe" cultures, respectively, with $r = 0.79$ and 0.89, both statistically significant at $P = 0.01$.

TABLE II

IRON AND CHLOROPHYLL PER GRAM OF FRESH WEIGHT IN DIFFERENT SECTIONS OF ACTIVE D LEAVES AT 20 AND 60 WEEKS OF PLANT GROWTH IN "PLUS Fe" AND "MINUS Fe" SOLUTION CULTURES

D Leaf Sections	20 weeks				60 weeks			
	Plus Fe		Minus Fe		Plus Fe		Minus Fe	
	Fe	Chlorophyll	Fe	Chlorophyll	Fe	Chlorophyll	Fe	Chlorophyll
	µgm	*mg*	*µgm*	*mg*	*µgm*	*mg*	*µgm*	*mg*
2	0.93	0.33	0.35	0.23	2.70	0.14	1.03	0.06
3	0.98	0.67	0.65	0.45	3.15	0.49	1.15	0.12
4	1.75	0.70	0.90	0.50	3.90	0.62	1.62	0.15
5	1.90	0.79	1.15	0.66	3.90	0.70	1.48	0.18
Av.	1.60	0.63	0.76	0.46	3.40	0.40	1.32	0.13

PROTEIN: The existence of a chlorophyll-protein compound in the chloroplast which Stoll named chloroplastin, according to Rabinowitch (11), had been postulated for a long time. According to Menke (7, 8), total leaf proteins are composed of approximately 2/3 cytoplasmic- and 1/3 chloroplastic-proteins. The results show (fig 3) that total protein concentrations increased from the proximal, No. 2, to the distal, No. 5, D leaf sections and they were higher in the "Plus Fe" than in the "Minus Fe" cultures. The curves for the concentrations of protein, chlorophyll and carotenoids (fig 4) appear to be similar in shape, although of different magnitude, and suggest possible interdependence in the genesis and in the functional activity of all three substances. Average concentrations of total proteins in D leaves for 75 weeks of plant growth were 7.04 and 5.48 mg/gm fresh tissue for the "Plus Fe" and "Minus Fe" cultures, respectively, (table III).

The average concentrations of chloroplastic proteins, according to Menke's formula (7, 8), were 2.66 and 2.06 mg/gm fresh weight, respectively, for the "Plus Fe" and "Minus Fe" cultures, that is, 1.29 times as great for the former as for the latter (table III). In the "Plus Fe" cultures, the ratios of chlorophyll to chloroplastic protein fluctuated from 19.1 to 19.8 between the chlorophyllous sections 3, 4 and 5, with an average, including section 3, of 17.5 parts of chlorophyll to 100 parts of protein (fig 5). Smith (31, 32, 33), who considers chlorophyll as the prosthetic group of a protein, found ratios of 16 parts of chlorophyll to 100 parts of protein. This ratio does not differ greatly from 17.5 to 100, that obtained for the "Plus Fe" cultures. In the "Minus Fe" cultures, the ratios of chlorophyll to chloroplastic protein 13.8, 12.1 and 9.9 for the leaf sections 3, 4 and 5, respectively, are inversely related to the chronological age of the tissues of the respective sections (fig 5). It is possible that such an inverse relationship resulted from the lysis of the bonds between chlorophyll and chloroplastic proteins with subsequent decomposition of the former and accumulation of the latter in tissues of advanced chronological age.

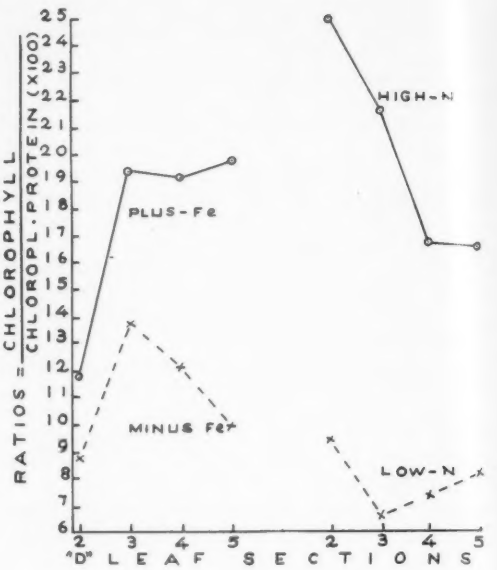


FIG. 5. Ratios of chlorophyll to 100 parts of chloroplastic protein in "Plus Fe" and "Minus Fe" and in "High N" and "Low N" cultures.

Chlorophyll and protein were correlated in the "Plus Fe" and "Minus Fe" cultures, with $r = 0.80$ and 0.79, respectively, both statistically significant at $P = 0.01$.

SUPPLEMENTARY RESULTS

Nitrogen plays a role equally as important as iron

TABLE III

AVERAGE CONCENTRATIONS PER GRAM OF FRESH WEIGHT, FOR 75 WEEKS OF PLANT GROWTH, OF TOTAL PROTEIN (N × 6.25), TOTAL ORGANIC NITROGEN, CAROTENOIDS, CHLOROPLASTIC PROTEIN (1/3 TOTAL PROTEIN), CHLOROPHYLL, RATIOS OF CHLOROPHYLL TO CHLOROPLASTIC PROTEIN (CHLOROPHYLL × 100/CHLOROPLASTIC PROTEIN) IN SECTIONS 1, 2, 3, 4 AND 5 OF D LEAVES OF "PLUS Fe" AND "MINUS Fe" SOLUTION CULTURES

D LEAF SECTIONS	TOTAL PROTEIN		TOTAL ORGANIC NITROGEN		CAROTENOIDS		"PLUS Fe"			"MINUS Fe"		
	+Fe	−Fe	+Fe	−Fe	+Fe	−Fe	CHLOROPHYLL	CHLOROPLASTIC PROTEIN	RATIOS	CHLOROPHYLL	CHLOROPLASTIC PROTEIN	RATIOS
	mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*		*mg*	*mg*	
1	3.41	2.70	0.90	0.79	....	....	0	...	...	0	...	...
2	4.54	3.53	1.06	0.86	0.024	0.014	0.180	1.52	11.8	0.103	1.18	8.7
3	7.53	5.42	1.78	1.33	0.085	0.043	0.490	2.52	19.4	0.250	1.81	13.8
4	9.44	7.05	2.37	1.77	0.118	0.060	0.605	3.17	19.1	0.285	2.35	12.1
5	10.25	8.68	2.50	1.63	0.148	0.065	0.680	3.43	19.8	0.286	2.90	9.9
Av.	7.04	5.48	1.72	1.28	0.094	0.045	0.488	2.66	17.5	0.231	2.06	11.1
+Fe/−Fe	1.28		1.34		2.09		2.11	1.29				

in the development of chlorophyll. Chlorophyll measurements were made in 3 lots of plants grown in a complete nutrient solution from September 1935 to July 1936. Then, from July to December 1936, each lot was transferred to solution cultures designated "Complete," i.e., with all the essential elements, "Minus N," i.e., without nitrogen, and "Minus Fe," i.e., without iron.

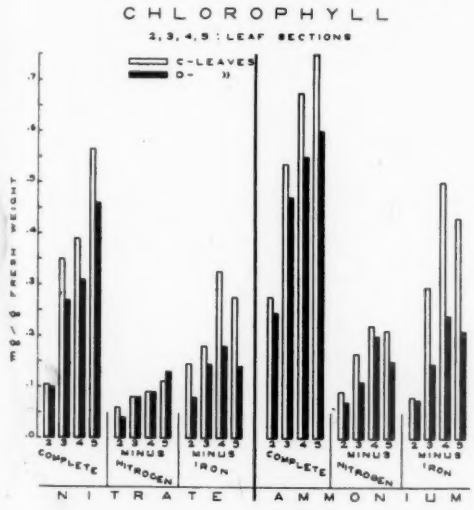
In December 1936, the average chlorophyll concentrations of the combined No. 2, 3, 4 and 5 sections of the mature C leaves in the "Minus N" cultures were as high as 25 % and in the "Minus Fe" cultures as high as 85 % as in the "Complete" cultures (fig 6). The concentrations of chlorophyll in the combined No. 2, 3, 4 and 5 of the active D leaves were in the "Minus N" cultures as high as 31 % and in the "Minus Fe" cultures as high as 58 % as in the "Complete" cultures. Hence, the losses of chlorophyll from N-deficiency in the "Minus N" cultures were approximately the same for both the chronologically more advanced C and the younger D leaves, because during the period of nitrogen deficiency, the chloroplastic proteins of the C leaves ceased to increase and, moreover, they underwent partial breakdown to release nitrogen for the formation of chloroplastic proteins in D leaves. Protein breakdown which was followed by chlorophyll breakdown was observed by Rombeck (14) in plants deficient in CO_2.

In the "Minus Fe" cultures, average chlorophyll concentrations in the combined sections were 2.8 times as great in C as in D leaves, indicating that chlorophyll and the associated chloroplastic proteins were produced in smaller amounts in D leaves during the period of iron deficiency, while in C leaves both substances remained only at slightly lower levels after the period of iron deficiency than before (fig 6). The data, also, suggest that iron translocation from old to new leaves, if any, was much lower than the requirements for chlorophyll synthesis in new tissues.

Additional results from pineapple plants, grown for 11 months in solution cultures with 84.0 and 7.0 mg of N/l of solution and designated as "High N" and "Low N" cultures, respectively, showed differences in final plant weights and in certain tissue constituents (fig 7). In the "High N" cultures the concentrations of chlorophyll and of total protein-N were as great as 3.5 and 1.5 times, respectively, as in the "Low N" cultures.

In the combined sections Nos, 3, 4 and 5 of D leaves, the ratios of chlorophyll to chloroplastic protein, the latter calculated according to Menke (7, 8) were 18.4 and 7.3 parts of chlorophyll to 100 parts of protein for the "High N" and "Low N" cultures, respectively (fig 5). The levels of the ratios of chlorophyll to chloroplastic protein (fig 5) in the "Plus Fe" cultures compared satisfactorily with those in the "High N" cultures. Also, the levels of similar ratios in the "Minus Fe" cultures compared favorably with those in the "Low N" cultures. This similarity in the ratios of chlorophyll to chloroplastic protein between the iron and nitrogen cultures indicate that both elements are equally important qualitatively, if not quantitatively, in the processes for the formation of chloroplastic protein and chlorophyll.

Intake of Mineral Nutrients: Since the metabolism of pineapple plants, as measured by plant and fruit weights, undergoes certain modifications with the changes in the concentrations of chlorophyll, it is natural to expect comparable changes in the rates of intake and assimilation of mineral nutrients.



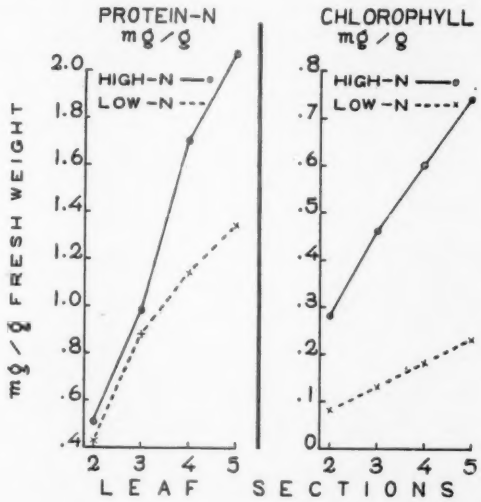


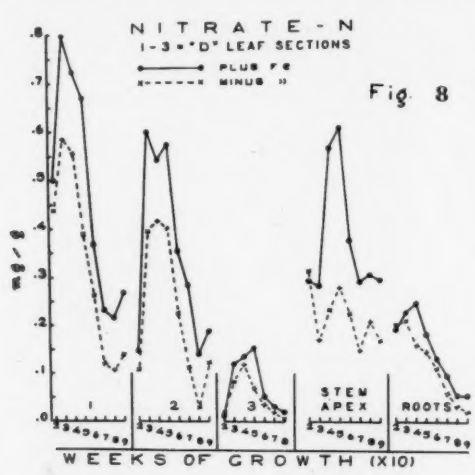
Fig. 6 (*left*). Chlorophyll in sections 2, 3, 4 and 5 of mature C and active D leaves of pineapple plants grown in "Complete," "Plus Fe" and "Minus Fe" cultures with nitrate and ammonium salts as nitrogen sources.

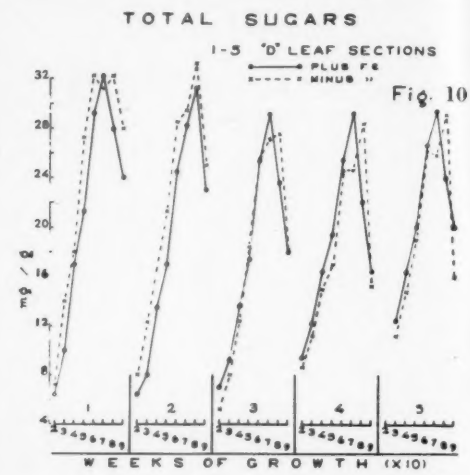
Fig. 7 (*right*). Chlorophyll and protein-N in sections 2, 3, 4 and 5 of D leaves grown in solution cultures with "High N" (84 mg N/l) and "Low N" (7 mg N/l).

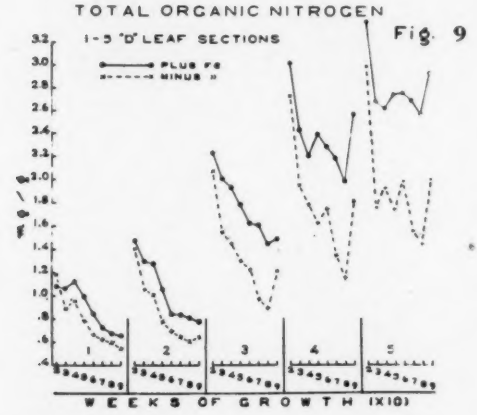
Measurements of nutrient elements in different sections of D leaves and in other plant parts, reported as average concentrations for 60 weeks of plant growth (table I), indicate that the intake of nitrate, calcium and magnesium was higher and of potassium and phosphorus lower in the "Plus Fe" than in the "Minus Fe" cultures. The high concentrations of potassium in the "Minus Fe" cultures resulted from concentration effects in view of smaller plant weights, which were 25 % smaller than plants in the "Plus Fe" cultures. A similar correction for plant weight differences applied to the concentrations of phosphorus would make equal the total intake of this element for both cultures and would greatly increase the average concentrations of calcium, magnesium iron and nitrate in the "Plus Fe" cultures.

The rates of nitrate-N, as indicated by the relative concentrations in sections 1, and 2 of D leaves (fig 8), was approximately 1.5 times as great in the "Plus Fe" as in the "Minus Fe" cultures. Nitrate concentrations greatly decreased in section 3 and dropped to almost zero values in 4 and 5 because of rapid assimilation in the chlorophyllous tissues.

The higher concentrations of nitrate in the non-chlorophyllous sections of the D leaves in the "Plus Fe" cultures should be attributed to a greater meta-







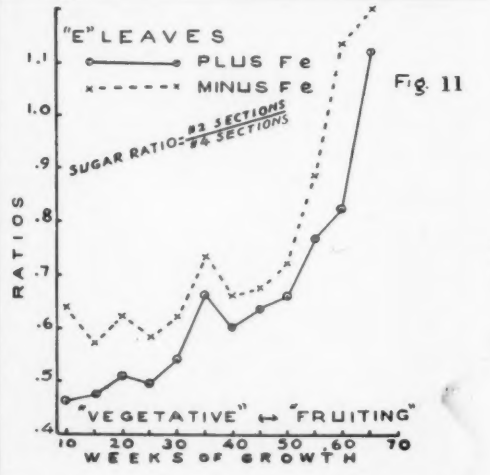


Fig. 8. Nitrate concentrations in sections 1, 2 and 3 of D leaves, stem apex and roots of "Plus Fe" and "Minus Fe" cultures.

Fig. 9. Total organic-N concentration in D leaf sections 1, 2, 3, 4 and 5 of "Plus Fe" and "Minus Fe" cultures.

Fig. 10. Total sugar concentrations in D leaf sections 1, 2, 3, 4 and 5 of "Plus Fe" and "Minus Fe" cultures.

Fig. 11. Ratios of total sugars in the proximal No. 2 to the distal No. 4 sections of young E leaves of "Plus Fe" and "Minus Fe" cultures.

bolic activity that presumably resulted from greater concentrations of chlorophyll (fig 8). In addition, the roots in the "Plus Fe" cultures possessed a greater permeability to solutes, including nitrate, presumably, because the surface areas of white tissue near the tip were considerably more extensive than in the "Minus Fe" cultures. Although total root weights per plant were 1.1 times as high in the "Minus Fe" as in the "Plus Fe" cultures, the average number of the roots was greater and their length shorter in the latter than in the former. Previous results (17, 18, 19, 25, 30) disclosed that pineapple plants grown in iron-deficient solution cultures produced few but very long roots with very small areas of white tissue. In cultures with ample iron and nitrogen in the nutrient solution, similar plants produced many but relatively shorter roots with greatly increased areas of white tissue near the tip. Therefore, the intake of nitrate at greater rates by the "Plus Fe" than the "Minus Fe" cultures presumably resulted from the larger areas of white tissue at the root tip. With greater rates of nitrate intake in the "Plus Fe" cultures, the concentrations of total organic nitrogen were 1.34 and of protein, 1.28 times as great as in the "Minus Fe" cultures (table III).

Total organic nitrogen, composed mostly of protein-N, proteose-N, peptide-N, amino-N and amide-N, accumulated more in the distal, No. 3, 4 and 5 than in the proximal No. 1 and 2 sections of D leaves (fig 9). Average total organic-N concentrations were higher in the "Plus Fe" than in the "Minus Fe" cultures and resulted from greater rates of intake and assimilation of nitrate. The curves of total organic-N (fig 9) greatly resemble in shape the ones of protein (fig 4), presumably because the latter constitutes more than 50 % of the total amount of the former.

Carbohydrates: Carbohydrate synthesis, depending on photosynthesis in which chlorophyll plays a dominant role, is reduced in chlorotic plants in relative proportion with the intensity of chlorosis. Various workers (1, 2, 3, 6) have found that the rates of photosynthesis, as measured by the evolution of oxygen in Chlorella cultures, are closely related to the concentrations of chlorophyll. In this study the relationship of chlorophyll concentrations to photosynthesis was measured by total plant and fruit weights, in addition to the average concentrations of total sugars and starch. The results (fig 10) show that the concentrations of sugars in the chlorophyllous distal sections 3, 4, and 5 were greater for the "Plus Fe" than for the "Minus Fe" cultures, while in the nonchlorophyllous proximal sections 1 and 2, they were reversed (29).

Sugar accumulated at the proximal nonchlorophyllous leaf regions by translocation from the distal chlorophyllous regions of the same or of other adjacent leaves. In the proximal leaf regions of active D and young E leaves with meristematic tissues, sugar concentrations decreased more at high than low rates of growth, as revealed by a comparison of figures 1 and 10, because of utilization in the formation of new tissues. Hence, the ratios of sugar concentrations in the proximal to those in the distal regions of young E leaves were inversely related to the amounts of sugar utilized by the tissues (fig 11) and indicated that with advancing plant age the sugars increased because of decreased rates of utilization in new tissue growth. Such ratios were greater in the "Minus Fe" than in the "Plus Fe" cultures, because of lower rates of sugar utilization in plant growth in the former than in the latter. At approximately 50 weeks of plant growth, which coincided with the onset of floral differentiation and cessation of new leaf production, the ratios increased rapidly for both cultures, thus indicating an end to sugar utilization for the formation of new leaf tissues.

Average sugar concentrations were not related quantitatively to the concentrations of chlorophyll in D leaves. In the chlorophyllous section 3-5, sugar concentrations were 1.025 times and chlorophyll 2.15 times as great in the "Plus Fe" as in the "Minus Fe" cultures. Also, average starch concentrations in the stem tissues were 52.0 and 43.6 mg for the "Plus Fe" and "Minus Fe" cultures, respectively, that is, 1.19 times as great for the former as for the latter.

Depressed plant and fruit growth in chlorotic plants has been attributed to carbohydrate deficiency. Chlorophyll synthesis was very low, if not completely arrested during the period of iron depletion, which was effective in the last 3 to 4 weeks of each 5-week period of changing of solution cultures. However, both sugars and starch were produced in ample amounts by the chlorotic plants of the "Minus Fe" cultures, as reported (fig 10), and had they been utilized at greater rates at the meristematic tissues of the proximal leaf regions their concentrations would have not exceeded those of the green plants of the "Plus Fe" cultures. Hence, the evidence suggests that deficiencies other than carbohydrates had developed in the "Minus Fe" cultures which were responsible for decreased plant and fruit growth. Moreover, whatever the nature of the deficiencies in the "Minus Fe" cultures, they developed during the periods of total iron depletion and simultaneously with the cessation of chlorophyll synthesis.

Discussion

In the light of the results of the investigations reported above, chlorosis was induced in the chlorophyllous tissue of the leaves by deficiencies in iron or in nitrogen, because both elements are associated with the processes for the formation of chloroplastic protein and chlorophyll. Rombeck (14) observed that deficiency in carbon dioxide in fast growing plants results in protein breakdown which is followed by chlorophyll breakdown.

The amounts of iron and nitrogen required by plants for the formation of chlorophyll and chloroplastic proteins depend on the rates of leaf expansion, which vary with different stages of growth and development and with the nutritional and climatic conditions in the environment.

It is quite evident in the results, reported above, that chlorophyll concentrations in the tissues increased with greater amounts of chlorophyll. However, there was no stoichiometric relationship between chlorophyll or chloroplastic protein and iron. Oserkowsky (11) found that only *active* iron but not total iron in the leaves of pear trees correlated with the amounts of chlorophyll. He (10) also found that cholorophyll correlated with the carotenoid content of the same pear leaf tissue.

The rate of photosynthesis in plants with variable chlorophyll concentrations, resulting from iron or nitrogen deficiencies, is proportional to the chlorophyll content, as measured by the evolution of oxygen by Chlorella cultures (1, 2). In plants which are chlorotic due to magnesium deficiency, the evidence indicates that magnesium plays a part in photosynthesis in addition to its effect upon chlorophyll content (3, 6).

Photosynthetic rates, as indicated by the quotient $PQ = \Delta O_2/\Delta CO_2$, do not allow one to identify the products of photosynthesis as simple sugars, or their polymers. In higher plants, measurement of total organic matter, as indicated by plant weights, is not a more reliable measure of photosynthetic activity than any of the other products, because the net increase is the resultant of the photosynthetic process minus the respiration process. In this study (fig 1), the combined average weights of the plants and fruits at the end of 75 weeks of growth were approximately 10.3 kgm for the "Plus Fe" and 7.6 kgm for the "Minus Fe" cultures. The separate average weights of the plants were 7.35 kgm and 6.10 kgm and of the fruits 2.95 kgm and 1.50 kgm, respectively, for the "Plus Fe" and the "Minus Fe" cultures. Hence, plant weights were 1.2 and fruit weights 2.0 times as great in the "Plus Fe" as in the "Minus Fe" cultures. It appears that chlorophyll concentrations were more closely related to fruit than to total plant weights or to sugar and starch concentrations.

In view of the overwhelming evidence in favor of increased photosynthetic rates with greater chlorophyll concentrations, the lack of a quantitative relationship between chlorophyll and sugar or starch concentrations should be attributed to the unsuitability of the experimental material for precise control of all the processes involved in photosynthesis.

Willstätter and Stoll (34) observed that the rate of photosynthesis as a function of chlorophyll and light intensities and temperatures failed to reveal, in their measurements of CO_2 assimilation by higher plants, any regular relationship between rate of photosynthesis and chlorophyll content. Emerson (1, 2), commenting of the results of Willstätter and Stoll, pointed out that the choice of leaves as material for a study of this kind was unfortunate, presumably as compared to Chlorella, because of the large number of variables introduced. Müller and Larsen (9) have measured twice as high rates of photosynthesis in *Sinapis alba* at 15,000 lux and equal chlorophyll concentrations when the nitrogen content of the leaves was 7 mg than 3.5 mg/50 cm^2. Pirson (12) observed, likewise, a nitrate effect on photosynthesis, which was not dependent on chlorophyll concentrations, by an immediate rise in the rate of photosynthesis following the addition of nitrate to a nitrate-deficient medium. Our interpretation of the various manifestations of plant activity in relation to chlorophyll or sugar concentrations or to plant and fruit weights is hampered by our limited knowledge of the functions of the different pigments and their associated proteins and of other substances in the cell. The recent finding of Shemin and his co-workers (16, 35), revealing that protoporphyrin IX, the basic structure of the molecules of chlorophyll, cytochroms, peroxidases, etc., is formed in the tricarboxylic acid cycle by the reaction of "active" succinate with glycine, show our heretofore limited knowledge in the generation and operation of the photosynthetic system.

In recapitulation, the effects of partial iron deficiency, as practiced in the "Minus Fe" cultures, caused an intake of one-half as much iron as in the "Plus Fe" cultures. Although the differences in the concentrations of iron that entered the chlorophyllous tissues were great, the absolute amounts were relatively small. Nevertheless, such differences stimulated in the "Plus Fe" cultures the production of almost twice as great amounts of chlorophyll, carotenoids and fruit weights, as in the "Minus Fe" cultures. Also, nitrate intake, total protein, chloroplastic protein and plant weights were, respectively, 1.5, 1.28, 1.29 and 1.21 times as great in the "Plus Fe" as in the "Minus Fe" cultures. The average concentrations of total sugars for entire D leaves and starch for entire stems were approximately the same for both cultures. There were certain differences in the concentrations of sugars between the proximal and distal sections of leaves, which resulted from different rates of synthesis and utilization.

Although the effects of iron in plant metabolism are ascertainable as differences in chlorophyll, carotenoids, chloroplastic proteins, etc., the mode of activation of this physiological mechanism by iron still remains a matter for speculation.

Symptomatologically, the chlorosis resulting from partial deficiency of iron or nitrogen is alike, although its development in nitrogen deficiency is more extensive and rapid than in iron deficiency. In both forms of chlorosis, the concentrations of chlorophyll are quantitatively related to the ones of carotenoids and chloroplastic proteins. Likewise, the rates of other metabolic activities fluctuated in relation to the concentrations of chlorophyll. The results suggest that chlorophyll, carotenoids and chloroplastic proteins are links in the same physiological system which operates by the combined activity of all three components.

Summary

Pineapple plants grown in solution cultures with ample and low iron, the latter within the limits of partial deficiency, contained more chlorophyll and produced greater plant and fruit weight in the cultures

with ample than deficient iron. The iron requirements of pineapple plants for chlorophyll synthesis were very low. Iron intake and translocation was reduced by phosphate through precipitation.

The symptoms of chlorosis did not differ between nitrogen-deficient and iron-deficient plants. During nitrogen deficiency, the leaves formed before and during the period of deficiency become chlorotic in variable degrees, the former by the breaking down of the chloroplasts in a medium lacking new nitrogen supplies for the regeneration of chloroplastic proteins and the latter for their initial formation. In iron deficiency, only the leaves formed during the deficiency period become chlorotic, while those formed previous to this period remain relatively green. Chlorosis in iron deficient plants develops when there is an inverse relationship between the rates of iron intake and plant growth. Iron accumulations, resulting long after the tissues have undergone functional differentiation, were not closely related to chlorophyll concentrations. Chlorosis resulting from iron or nitrogen deficiencies differed more in extent than in quality. It was more extensive and rapid in plants with nitrogen than with iron deficiency.

Chlorophyll, carotenoids and protein increased simultaneously in the green leaves of plants with iron sufficiency, but decreased in the chlorotic leaves with iron deficiency.

Sugar concentrations in the proximal regions of the leaves with meristematic tissues were greater in chlorotic than in green leaves, because of low sugar utilization at low growth rates. Plant or fruit growth rates were not related to total sugar or starch concentrations.

Nitrate intake and assimilation was greater in green than in chlorotic plants and was responsible for the greater amounts of total organic-N and protein in the former than in the latter.

Chlorophyll concentrations were correlated with the amounts of iron and nitrogen. Plants with ample nitrogen contained 1.5 times as much protein and 3.5 times as much chlorophyll as plants with low nitrogen. Chlorophyll concentrations were 2.15 and fruit weights 2.00 times as great in plants with ample "Plus Fe" as in deficient "Minus Fe" cultures.

Literature Cited

1. Emerson, R. The relation between the maximum rate of photosynthesis and concentration of chlorophyll. Jour. Gen. Physiol. 12: 609–622. 1929.
2. Emerson, R. Photosynthesis as a function of light intensity and of temperature with different concentrations of chlorophyll. Jour. Gen. Physiol. 12: 623–639. 1929.
3. Fleischer, Walter The relation between chlorophyll content and rate of photosynthesis. Jour. Gen. Physiol. 18: 573–597. 1955.
4. Guthrie, J. D. A stable colorimetric standard for chlorophyll determinations. Amer. Jour. Bot. 15: 86–87. 1928.
5. Johnson, M. O. Manganese chlorosis of pineapples: Its cause and control. Agr. Expt. Sta., Hawaii, Bull. 52: 1–38. 1924.
6. Kennedy, S. K. The influence of magnesium deficiency, chlorophyll concentration, and heat treatment on the rate of photosynthesis of Chlorella. Amer. Jour. Bot. 27: 68–73. 1940.
7. Menke, W. Untersuchungen über das Protoplasma grüner Pflanzenzellen. I. Isolierung von Chloroplasten aus Spinätblattern. Zeits. Physiol. Chem. 257: 43–48. 1939.
8. Menke, W. Untersuchungen über das Protoplasma grüner Pflanzenzellen. II. Der chlorophyllgehalt der Chloroplasten aus Spinatblättern. Zeits. Physiol. Chem. 263: 100–103. 1940.
9. Müller, D. and Larsen, P. Analyse der Stoff produktion bei Stickstoff und Kalimangel. Planta 33: 501–517. 1935.
10. Oserkowsky, J. Relation between the green and the yellow pigments in chlorotic leaves. Plant Physiol. 7: 711–716. 1932.
11. Oserkowsky, J. Quantitative relation between chlorophyll and iron in green and chlorotic pear leaves. Plant Physiol. 8: 449–468. 1933.
12. Pirson, André Ernährungs- und Stoff-wechselphysiologische Untersuchungen an Fontinalis und Chlorella. Zeits. Bot. 31: 193–267. 1937.
13. Rabinowitch, E. I. Photosynthesis, vol. I. Pp. 1–599. Interscience Publ., New York 1945.
14. Rombeck, Franz The metabolism of green leaves in light in a carbon dioxide-free atmosphere. Jahrb. wiss. Bot. 93: 187–241. 1943. (Chem. Abstr. 39: 4353).
15. Schertz, F. M. The quantitative determination of chlorophyll. Plant Physiol. 3: 211–216. 1928.
16. Shemin, D., Russell, C. S. and Abramsky, T. The succinate-glycine cycle. I. Mechanism of pyrrole synthesis. Jour. Biol. Chem. 215: 613–626. 1955.
17. Sideris, C. P. Behavior of pineapple plants in water cultures of varying hydrogen ion concentrations. Expt. Sta. Assoc. Hawaiian Pineapple Canners Bull. 4: 1–15. 1926. (Private).
18. Sideris, C. P. et Krauss, B. H. Carences minérales des plantes: le rôle physiologique du fer, du titane, du manganèse, du bore et du fluor sur le développement de l'*Ananas sativus* et du *Zea Mays*. Congr. intern. pathol. compar. 2e Congr., Paris 2: 416–432. 1931.
19. Sideris, C. P. and Krauss, B. H. The effect of different salts and concentrations of iron on the growth of pineapple plants. Pineapple Quart. 2: 64–71. 1932. (Private).
20. Sideris, C. P. and Krauss, B. H. The growth of pineapple plants in complete water cultures either with ammonia or nitrate salts. Growth 1: 204–210. 1937.
21. Sideris, C. P., Krauss, B. H. and Young, H. Y. Assimilation of ammonium and nitrate nitrogen from solution cultures by roots of *Pandanus veitchii* Hort., and distribution of the various nitrogen fractions and sugars in the stele and cortex. Plant Physiol. 12: 899–928. 1937.
22. Sideris, C. P., Krauss, B. H. and Young, H. Y. Assimilation of ammonium and nitrate by pineapple plants grown in nutrient solutions and its effects on nitrogenous and carbohydrate constituents. Plant Physiol. 13: 489–527. 1938.
23. Sideris, C. P. and Krauss, B. H. Growth phenomena of pineapple fruits. Growth 2: 181–196. 1938.
24. Sideris, C. P., Young, H. Y. and Chun, H. H. Im-

provements in the determination of iron by the Nitroso-R Salt Method. Ind. Eng. Chem., Anal. Ed. 16: 272. 1944.
25. SIDERIS, C. P. and YOUNG, H. Y. Effects of iron on chlorophyllous pigments, ascorbic acid, acidity and carbohydrates of *Ananas comosus* (L.) Merr. supplied with nitrate or ammonium salts. Plant Physiol. 19: 52–75. 1944.
26. SIDERIS, C. P. and YOUNG, H. Y. Effects of potassium on chlorophyll, acidity, ascorbic acid and carbohydrates of *Ananas comosus* (L.) Merr. Plant Physiol. 20: 649–670. 1945.
27. SIDERIS, C. P. and YOUNG, H. Y. Effects of nitrogen on chlorophyll, acidity, ascorbic acid, and carbohydrate fractions of *Ananas comosus* (L.) Merr. Plant Physiol. 22: 97–116. 1947.
28. SIDERIS, C. P. and YOUNG, H. Y. Growth of *Ananas comosus* (L.) Merr., at different levels of mineral nutrition under greenhouse and field conditions. I. Plant and fruit weights and absorption of nitrate and potassium at different growth intervals. Plant Physiol. 25: 594–616. 1950.
29. SIDERIS, C. P. and YOUNG, H. Y. Growth of *Ananas comosus* (L.) Merr., at different levels of mineral nutrition under greenhouse and field conditions. II. Chemical composition of the tissues at different growth intervals. Plant Physiol. 26: 456–474. 1951.
30. SIDERIS, C. P. Iron in pineapple growth and nitrogen utilization. Research Report 34: 1–27. 1955. (Private).
31. SMITH, E. L. Solutions of chlorophyll-protein compounds (phyllochlorins) extracted from spinach. Science 88: 170–171. 1938.
32. SMITH, E. L. Chlorophyll as the prosthetic group of a protein in the green leaf. Science 91: 199–200. 1940.
33. SMITH, E. L. The chlorophyll-protein compound of the green leaf. Jour. Gen. Physiol. 24: 565–582. 1941.
34. WILLSTÄTTER, R. and STOLL, A. Untersuchungen über die Assimilation die Kohlensaüre. Pp. 1–424. Springer, Berlin, 1918.
35. WRISTON, J. C., JR., LACK, L. and SHEMIN, D. The mechanism of porphyrin formation. Further evidence on the relationship of the citric acid cycle and porphyrin formation. Jour. Biol. Chem. 215: 603–611. 1955.

KINETICS OF SULFATE ABSORPTION BY BARLEY ROOTS[1]

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This paper reports the results of short-term experiments on the absorption, by excised barley roots, of sulfate labeled with S^{35}. The findings are consistent with the view that the absorption of sulfate is mediated by carriers to which the ions are bound in their passage across a barrier impermeable to free sulfate ions. As regards kinetics and specificity, the results are interpreted in terms of the enzyme-kinetic hypothesis of the operation of carriers, as given by Epstein and Hagen (6).

MATERIALS AND METHODS

RADIOACTIVE SULFATE: To a 10-ml aliquot of a stock solution of K_2SO_4 was added carrier-free radiosulfate in dilute HCl, obtained from the Oak Ridge National Laboratory. The tagged solution was evaporated to dryness to free it from HCl, made to the original volume (10 ml) with water, and added to the stock solution of K_2SO_4. The specific activity was about 2 $\mu c/\mu eq$ SO_4.

SELENATE: Commercially available "CP" potassium selenate was found to be contaminated with selenite. The selenate was reduced to selenium with hydroxylamine hydrochloride, the selenium washed, dissolved in nitric acid, and after neutralization of the solution, oxidized to selenate with bromine, essentially according to the method of Dennis and Koller (1). The stock solution of selenic acid was titrated with standard KOH to pH 5.5. The concentration of the stock solution was determined 1) from the amount of KOH added, 2) by evaporating an aliquot of the stock solution of K_2SeO_4 and weighing the salt, and 3) by reduction of selenate and weighing the selenium, according to Hillebrand, Lundell, Bright, and Hoffman (9). The results of all three methods agreed within 2 %. Selenite was less—probably much less—than 0.1 percent of the selenate, on an equivalent basis, according to the test of Dennis and Koller (1).

ASSAY FOR RADIOSULFATE: After an absorption experiment, the 1.00-gm (fresh weight) samples of excised roots were transferred to 30-ml Pyrex beakers. About 3 ml ethyl alcohol, 1 ml 0.1 *N* K_2SO_4 (nonradioactive), and 1 ml $Mg(NO_3)_2$ reagent (14) were added, the solutions evaporated to dryness, and the samples ashed overnight at 500° C. The ash was moistened, acidified with HCl, and the clear solution evaporated to dryness. The chloride salt was dissolved by adding 10 ml 0.35 M acetic acid at about 70° C. Benzidine sulfate was precipitated by addition of 2 ml benzidine reagent (8.0 gm benzidine di-hydrochloride dissolved in 500 ml water containing 5 ml 1 *N* HCl, filtered through #42 Whatman filter paper before using). After addition of 4 ml acetone, the beakers were tightly covered with aluminum foil and placed in the refrigerator at 8° C overnight. The suspension of benzidine sulfate was filtered, under suction, through circles of #1 Whatman filter paper, placed in the bottom of ¼-ounce seamless tin boxes (made by the Buckeye Stamping Company, Columbus, Ohio), 3.3 cm in diameter and 8 mm deep, the bottom

[1] Received December 29, 1955.

being perforated by 25 evenly spaced 1 mm holes. The beaker and the precipitate on the filter-funnel were rinsed with 2 ml cold acetone, whereupon the samples were ready for counting. A thin window (2.4 mg/cm^2) Geiger-Müller tube and conventional scaling circuit were used. Aliquots of the radiosulfate solutions used in the experiments were taken through the entire procedure outlined above to serve as standards in calculating radiosulfate absorbed by the root samples. The above method for precipitating benzidine sulfate is a modification of that given by Kahn and Leiboff (11).

Excised Barley Roots: Seeds of barley, var. Atlas 46, were germinated and seedlings grown as described earlier (5). After soaking in aerated water for 24 hours the seeds were planted over an aerated solution of $Ca(H_2PO_4)_2$, 2×10^{-4} M, and seedlings grown for 5 days, during which time the solution was renewed once. During the entire period the plants were kept in a dark chamber at 24° C. Just before the experiment proper, the roots were rinsed, excised, and suspended in 4 liters of water.

Standard Experimental Procedure: The procedure of conducting the absorption experiments proper was essentially as described before (7). Excised roots were blotted, 1.00-gm portions weighed out and transferred to 100-ml test tubes containing 50 ml water. The absorption period was initiated by decanting the water and replacing it with 50 ml of the experimental solution, adjusted to pH 4.0 unless otherwise stated. After the absorption period, the solution was decanted and replaced by a solution of non-radioactive $CaSO_4$, 1 meq/l, or, in some experiments, by water, to free the roots of the diffusible fraction of sulfate in the "outer" space (5). During both absorption and desorption periods, the tubes were kept in a water bath at 30° C, and the solutions were constantly aerated.

Results

Sulfate absorption was influenced by the pH of the test solutions. Highest absorption rates were observed from solutions adjusted to pH 4. The test solutions were not buffered, but solutions adjusted to pH 4.0 with HCl did not change by more than 0.2 unit during a 3-hour absorption period. All subsequent experiments were conducted at pH 4. After a period of absorption of S^*O_4,[2] the total S^*O_4 within the root can be cleanly separated into two fractions, 1) diffusible and 2) actively absorbed S^*O_4. This is demonstrated in figure 1. Before zero time, the roots were exposed to a solution of $K_2S^*O_4$, 0.5 meq/l, for an absorption period of 60 min. Root samples were then blotted, for determination of total labeled sulfate, and others exposed to water or .1 meq/l solutions of non-radioactive $CaSO_4$ for desorption periods ranging up to 120 min. A rapid loss of S^*O_4 from the roots is followed by a period in which there is no further loss of S^*O_4, either to water or solutions of $CaSO_4$. The loss is due to diffusion of S^*O_4 from the "outer" space of the tissue, i.e., a space to which inorganic ions have free and reversible access by diffusion

[2] Asterisks indicate radioactive labeling.

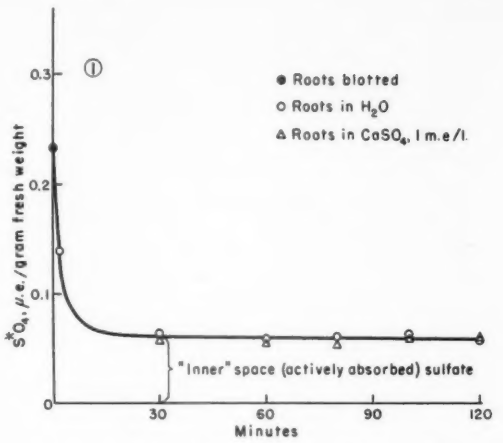


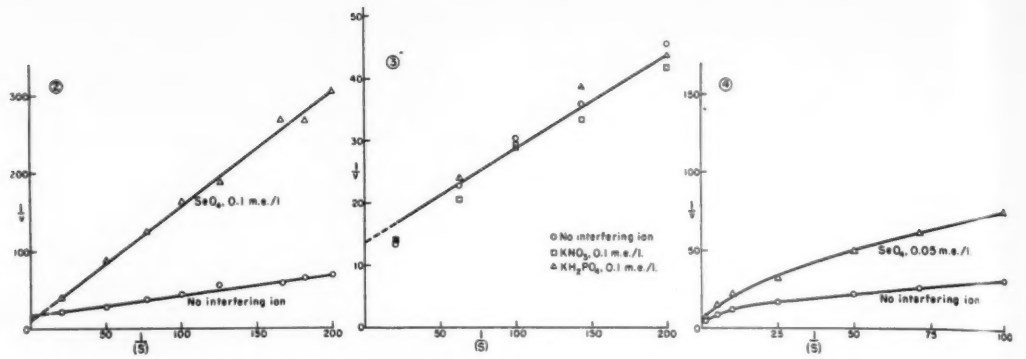
Fig. 1. Loss of labeled sulfate by excised barley roots. Before zero time, the roots had been exposed to $K_2S^*O_4$, 0.5 meq/l, for 60 min.

(5). The magnitude of the "outer" space, in these roots, is 0.23 ml/gm fresh weight (5). The fraction remaining after the rapid, initial loss has been absorbed by the metabolic, active transport mechanism, or in other words, has been transferred to the "inner" space. The present paper is concerned with this fraction only. For routine measurements of "inner" space, or actively absorbed sulfate, the roots, after the absorption period in S^*O_4 solutions, were exposed to solutions of $CaSO_4$, 1 meq/l, for a period of 60 min. All subsequent measurements refer to roots freed of the diffusible S^*O_4 fraction in this manner.[3] For determinations of the rate, v, of S^*O_4 absorption, 3-hr absorption periods were used. Absorption was a linear function of time, and the 3-hr value gave an adequate measurement of the rate of absorption.[4]

In figure 2, the reciprocal of the absorption rate, 1/v, has been plotted against the reciprocal of the substrate ion (S^*O_4) concentration, 1/(S) (cf Epstein and Hagen, 6). The S^*O_4 concentration, (S), was varied from 0.005 meq/l to 0.05 meq/l. Straight lines were obtained, evidence that the rate, v, of S^*O_4 absorption varied as a function of S^*O_4 concentration, (S), according to Michaelis-Menten kinetics (13). Selenate competitively interfered with S^*O_4 absorp-

[3] In the earlier experiments on absorption of bromide (2), complete removal of the diffusible bromide was not essential because the rate of active absorption of bromide is so high that diffusible bromide remaining in the "outer" space of the tissue, after 3 water rinses, represents a negligible proportion of the total measured uptake.

[4] When amounts absorbed were plotted against time, the straight line obtained extrapolated to a positive value at zero time, for the higher concentrations used. This introduces an error in our rate measurements never exceeding 10% of the total measured absorption. The nature of the sulfate binding which this value represents has not been further investigated.



FIGS. 2 to 4. Double reciprocal plots of the rate of absorption of S^*O_4 and the effects of other anions. The rate, v, of S^*O_4 absorption is in μeq/gm fresh wt × 3 hrs. The concentration, (S), of S^*O_4 in the solution is in meq/l.

FIG. 2. The effect of selenate; the concentration of S^*O_4 varied over the range 0.005 meq/l to 0.05 meq/l.

FIG. 3. The effects of nitrate and phosphate; the range of S^*O_4 concentrations was as in figure 2.

FIG. 4. The effect of selenate; the concentration of S^*O_4 varied over the range 0.01 meq/l to 1 meq/l.

tion, indicating identical binding sites on the carriers for these two ions. Neither nitrate nor phosphate, at a concentration of 0.1 meq/l, had a significant effect on the rate of S^*O_4 absorption (fig 3). Figure 4 shows that at S^*O_4 concentrations in excess of 0.05 meq/l, approximately, rates of absorption are higher than would be expected on the basis of the extrapolation indicated in figures 2 and 3.

Viets (18) has shown that calcium ions increase the rate of absorption of both monovalent cations and anions by excised barley roots. Tanada (16) made similar observations in experiments with excised mung bean roots. The rate of absorption of S^*O_4 was markedly increased by additions of $CaCl_2$ to the solution (fig 5). At a S^*O_4 concentration of 0.5 meq/l, maximal activation (to 179 % of the controls) was obtained at a $CaCl_2$ concentration of 3 meq/l. At 0.05 meq/l S^*O_4, a $CaCl_2$ concentration of 1 meq/l gave maximal activation, to 203 % of the control value. At higher concentrations of $CaCl_2$, the rate of S^*O_4 absorption declined somewhat. However, even at the highest concentration of $CaCl_2$ used, 10 meq/l, uptake of S^*O_4 from the 0.05 meq/l solution of S^*O_4 was still 172 % of the control. Under these conditions, the ratio Ca/S^*O_4 in the solution was 200/1, on an equivalent basis.

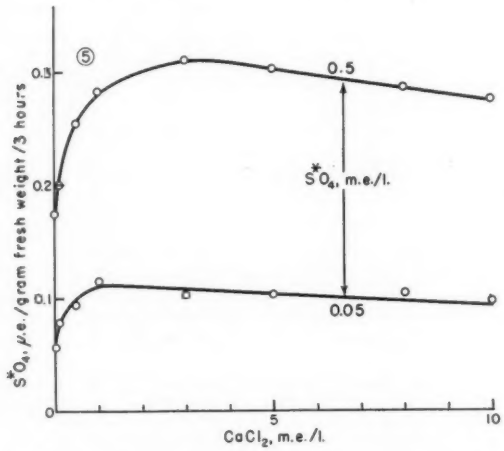


FIG. 5. The effect of calcium on the rate of S^*O_4 absorption.

DISCUSSION

The rate of absorption of sulfate was found to depend on the sulfate concentration in the manner expected for reactions following classical enzyme kinetics (13), for the concentration range 0.005 meq/l to 0.05 meq/l. The implication is that sulfate absorption proceeds in three steps (cf Epstein and Hagen, 6): 1) sulfate ions reversibly combine with reactive sites of carriers; 2) the carrier-sulfate complex traverses a membrane not permeable to free sulfate ions; 3) sulfate ions are released internally in a rate-limiting, essentially irreversible step.

Absorption of sulfate in the present experiments was followed by means of the radioactivity of S^{35}, after exposure of the roots to solutions of $K_2S^{35}O_4$. It is possible, therefore, that a fraction of what has here been called, for convenience, "inner" space or "absorbed" sulfate was not sulfate at all, but represented S^{35} incorporated into organic compounds. However, the subsequent fate of sulfate, after its absorption, is beyond the scope of this paper, which deals only with the rates at which sulfate is actively absorbed from the solution. These rate measurements lend themselves to interpretations of the mechanism of absorption, regardless of the eventual destinations of the absorbed sulfur atoms. Kylin (12) has demonstrated incorporation of S^{35} into organic fractions of wheat seedlings. The process was slow compared with the rate at which S^*O_4 was absorbed.

In the earlier experiments on the kinetics and

selectivity of ion absorption, the substrate ions whose rate of absorption was determined were bromide (2, 3), rubidium (6), and strontium (7), elements not normally present in significant amounts in the roots. Such elements were chosen to make sure that the measurements of absorption, made by means of radioactive tracing, represented values of net absorption. The radioactivity appearing in the roots could not be due to mere exchange of labeled ions for stable ones of the same chemical species initially present in the tissue, without any net change in the concentration of that ion in the tissue. Not so in the present instance. The measurements of absorption of labeled sulfate reported here may represent net absorption, or exchange with a pool of sulfur so large that the resulting isotopic dilution would render subsequent loss of radiosulfur to solutions of non-radioactive sulfate undetectable by our methods. The possibility of such exchange, emphasized by one of the reviewers of this paper, is being investigated. In either case, the absorption is an active, metabolic process. It is abolished by anaerobic conditions.[5]

Interference by other ions with the absorption of S^*O_4 was studied by exposing the roots to solutions of $K_2S^*O_4$ containing, in addition, the potassium salt of the ion whose effect on S^*O_4 absorption was to be investigated. Selenate competed with S^*O_4 (fig 2), but phosphate and nitrate were without effect (fig 3). The Michaelis constant for S^*O_4, i.e., the concentration giving half the maximal rate obtainable, was 0.019 meq/l. The calculated value for selenate, from the same experiment (fig 2) was 0.015, indicating that the affinity of these two ions for their common binding site is of the same order of magnitude.

At S^*O_4 concentrations above 0.05 meq/l approximately, the experimental points in the double-reciprocal plot (fig 4) fall below the values expected on the basis of the extrapolation indicated in figure 2, i.e., rates of absorption are higher than expected. The same phenomenon has been observed in absorption of rubidium in the presence of sodium (6) and in absorption of strontium (7). The implication is that at higher concentrations of the ions additional sites are involved which differ in their affinities for ions from the sites which mediate absorption at the lower concentrations.

The present demonstration of competition between sulfate and selenate for common sites which have little or no affinity for other ions (phosphate, nitrate, chloride) is the last in a series of investigations which have consistently revealed a high degree of specificity in the mutual effects of inorganic ions in the absorption process. The previous instances were 1) competition among potassium, rubidium, and cesium for sites having little affinity for sodium and lithium (6); 2) competition among bromide, chloride, and iodide, and lack of competition by nitrate for the halide binding sites (2, 3); 3) competition among calcium, strontium and barium for binding sites which have but little affinity for magnesium ions (7).

In all these cases, the specificities observed were concordant with previous evidence on ion selectivity and mutual interferences, as discussed by Epstein (4). The same is true in the present instance. Cases of sulfate-selenate antagonism have been described for wheat (10), corn (17), yeast (8), *Chlorella vulgaris* (15), and *Aspergillus niger* (19). The specificity of the carrier sites of the roots for sulfate and selenate is very high. Nitrate and phosphate failed to interfere with S^*O_4 absorption, although their concentration was 0.1 meq/l—20 times the lowest concentration of S^*O_4 tested (fig 3).

[5] Recent unpublished experiments by one of us (E. E.) indicate that the absorption of S^*O_4, measured by radioactive tracing, represents a net uptake rather than an exchange with native, non-radioactive sulfur.

Summary

The absorption, by excised barley roots, of sulfate labeled with S^{35} has been studied in experiments lasting 3 hours. Diffusible labeled sulfate present in the tissue at the end of the absorption period was removed by exposing the roots to solutions of non-radioactive sulfate for 1 hour. This procedure leaves for final measurement the actively absorbed labeled sulfate only.

Rates of absorption were highest at pH 4 and the experiments reported were done at this pH. The rates of sulfate absorption varied in response to the external sulfate concentration in a manner consistent with the view that the absorption of sulfate involves the attachment of the ions to specific sites of carriers. Selenate competed with sulfate on approximately even terms. Phosphate, nitrate, and chloride had no measurable affinity for the sulfate-selenate binding sites. Calcium increased the rate of sulfate absorption.

Literature Cited

1. Dennis, L. M. and Koller, J. P. Selenic acid and copper selenate. Jour. Amer. Chem. Soc. 41: 949–970. 1919.
2. Epstein, E. Mechanism of ion absorption by roots. Nature 171: 83–84. 1953.
3. Epstein, E. Ion absorption by plant roots. Proc. Ann. Oak Ridge Summer Symp. 4: 418–434. 1953.
4. Epstein, E. Absorption of ions by plant roots. In: Electrolytes in Biological Systems, A. M. Shanes, ed. Pp. 101–111. Amer. Physiol. Soc., Washington, D. C. 1955.
5. Epstein, E. Passive permeation and active transport of ions in plant roots. Plant Physiol. 30: 529–535. 1955.
6. Epstein, E. and Hagen, C. E. A kinetic study of the absorption of alkali cations by barley roots. Plant Physiol. 27: 457–474. 1952.
7. Epstein, E. and Leggett, J. E. The absorption of alkaline earth cations by barley roots: Kinetics and mechanism. Amer. Jour. Bot. 41: 785–791. 1954.
8. Fels, I. G. and Cheldelin, V. H. Selenate inhibition studies. III. The role of sulfate in selenate toxicity in yeast. Arch. Biochem. 22: 402–405. 1949.
9. Hillebrand, W. F., Lundell, G. E. F., Bright, H. A. and Hoffman, J. I. Applied inorganic analysis,

2nd ed. P. 336. John Wiley and Sons, Inc., New York 1953.
10. Hurd-Karrer, A. M. Relation of sulphate to selenium absorption by plants. Amer. Jour. Bot. 25: 666–675. 1938.
11. Kahn, B. S. and Leiboff, S. L. Colorimetric determination of inorganic sulfate in small amounts of urine. Jour. Biol. Chem. 80: 623–629. 1928.
12. Kylin, A. The uptake and metabolism of sulphate by deseeded wheat plants. Physiol. Plantarum 6: 775–795. 1953.
13. Lineweaver, H. and Burk, D. The determination of enzyme dissociation constants. Jour. Amer. Chem. Soc. 56: 658–666. 1934.
14. Richards, L. A., ed. Diagnosis and improvement of alkali soils. P. 129. U. S. Dept. Agr., Handbook No. 60. 1954.
15. Shrift, A. Sulfur-selenium antagonism. I. Antimetabolite action of selenate on the growth of *Chlorella vulgaris*. Amer. Jour. Bot. 41: 223–230. 1954.
16. Tanada, T. Effects of ultraviolet radiation and calcium and their interaction on salt absorption by excised mung bean roots. Plant Physiol. 30: 221–225. 1955.
17. Trelease, S. F. and Beath, O. A. Selenium. Pp. 1–292. Published by the authors, New York 1949.
18. Viets, F. G. Calcium and other polyvalent cations as accelerators of ion accumulation by excised barley roots. Plant Physiol. 19: 466–480. 1944.
19. Weissman, G. S. and Trelease, F. S. Influence of sulfur on the toxicity of selenium to Aspergillus. Amer. Jour. Bot. 42: 489–495. 1955.

PLASTID PIGMENT CHANGES IN THE EARLY SEEDLING LEAVES OF ZEA MAYS L.[1]

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Most publications dealing with the analyses and interrelationships of the plastid pigments of maize and Avena have been confined to groups of pigments rather than specific plastid pigments (3, 4, 5, 13). Since the changes in a group of pigments do not necessarily reflect the behavior of the individual pigments, knowledge about the specific pigment changes is desirable.

Moster et al (7, 8) have determined the kinds, as well as the amounts, of the major carotenoid pigments in young maize plants grown in the light and then exposed to different light intensities. However, information is lacking on the development of the specific carotenoid pigments in dark-grown corn seedlings and in etiolated corn seedlings exposed to light. This paper will present information on these subjects.

Methods

All seedlings were planted in quartz sand and grown in a constant temperature chamber equipped with fluorescent lamps. The culture methods, as well as a description of the light source and constant temperature chamber, have been described (9).

Leaf samples for analyses were obtained in the following manner: the second seedling leaf was removed from 15 different plants and the 15 leaves divided into 3 samples of 5 leaves each. Using a steel die, a section 7 by 52 mm was punched from the distal end of each leaf. These 5 leaf punches were pooled to form one sample. The distal end of the second leaf was selected because it was found to be fully expanded at the time the analyses were started. Thus the pigment content is actually based upon a constant area. However, in order to make the units of concentration more easily comparable to values found in the literature, all pigment concentrations have been expressed as μgm/gm of initial dry weight of leaf. This is a valid method of expressing the pigment content, since the initial dry weights of the leaf sections in the various experiments were not significantly different and there was less than a 2 % difference between the dry weights of leaf sections taken over a 7-day period.

Determinations of initial dry weights were made from material collected 6 days after planting. Three collections of 5 leaf punches were made in the same manner as collections for pigment analyses. Each sample of 5 leaf punches was dried at 75° C for 24 hours, weighed and the weights averaged to give the initial dry weight.

The carotenoid pigments were extracted and chromatographically separated on magnesia columns using methods similar to those employed by Strain (11). The leaf samples were immersed in hot water (90 to 100° C) for 3 minutes and ground in a mortar containing sand, 15 ml of methanol and 0.1 gm $CaCO_3$. The resulting brei was filtered through a medium sintered glass filter and the filtrate was transferred to a separatory funnel. The chlorophylls were saponified by treating the filtrate with 15 ml of methanol containing KOH (35 gm/100 ml). Thirty ml of a 1 : 1 (v/v) mixture of petroleum ether and ethyl ether was then added to the methanol solution and the mixture was shaken gently. The carotenoids were transferred to the petroleum ether–ethyl ether solution by the addition of 25 ml of saturated salt water accompanied by gentle shaking, followed by the addition of 10 to 20 ml of water with further gentle shaking. The methanol-water solution was drained into a second separatory funnel, further diluted with saturated salt water, and again extracted with 15 ml of a 1 : 1 mix-

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ture of petroleum ether and ethyl ether. The petroleum ether–ethyl ether extracts were combined and washed 4 times with water. These washings removed all traces of methanol and most of the ethyl ether leaving the carotenoids dissolved in petroleum ether containing a little ethyl ether. The carotenoid pigments were chromatographically separated by pouring the petroleum ether solution over a column (130 mm long and 11 mm in diameter) composed of a 1 : 1 (w/w) mixture of magnesia and Hyflo Supercel. A vacuum (about 26 cm of Hg pressure) was used to draw the solvent through the adsorbent. Within 10 minutes after the petroleum ether solution was poured over the column, two well separated colored zones appeared. The upper yellow zone consisted of a mixture of carotenols, while the lower orange zone was composed of a mixture of carotenes. Coincident with the time that the last of the petroleum ether passed into the column, a 2 : 1 (v/v) mixture of petroleum ether and ethyl ether was poured over the column. The carotenes passed rapidly (in about 10 min) through the column and were collected in the perculate. The perculate, containing the carotenes, was evaporated under reduced pressure to dryness and the carotenes redissolved in hexane. After prolonged washing (90 min) with the petroleum ether–ethyl ether solvent the yellow band of carotenols was resolved into 5 distinct bands: a yellow band, which extended 2 mm beneath the surface of the absorbent, a lemon yellow band 2 mm in width located about 5 mm beneath the top band, a yellow band 6 mm in width 10 mm beneath the second band, an orange-yellow band 20 mm in width 10 mm beneath the third band and a yellow band 12 mm in width 5 mm beneath the fourth band. Each colored band was cut from the extruded column and eluted with 95 % ethyl alcohol over a sintered glass filter. The filtrates were washed into 10-ml volumetric flasks, made to volume with 95 % ethanol and the absorption spectrum of each pigment determined. The absorption spectrum of the pigment component eluted from the top yellow band had no definite absorption peaks and when this pigment component was readsorbed on a calcium hydroxide column and washed with ethyl ether it was resolved into 5 distinct components. These pigments made up a very small part of the total carotenoids and were not further investigated. The 4 remaining colored bands proved to be single pigments which have been characterized by Strain (11). These 4 bands in the order of their position on the column, starting from the top, had properties which were the same as the carotenoids neoxanthin, flavoxanthin c, lutein and violaxanthin b, respectively. Readsorption of these pigments upon $CaCO_3$, $Ca(OH)_2$ and/or alumina columns followed by washing with suitable solvent systems failed to reveal any other colored contaminates.

The orange pigment component that passed completely through the magnesia column, and was subsequently dissolved in hexane, exhibited an absorption spectrum very much like that of β-carotene. However, when this pigment component was adsorbed on an alumina column and washed with hexane, it was resolved into two orange bands. The absorption spectra of the upper and lower bands, respectively, were found to be identical to the absorption spectra reported by Beadle and Zscheile (2) for β-carotene and neo-β-carotene isolated from spinach leaves. A β-carotene fraction was isolated from fresh spinach leaves by the method of Beadle and Zscheile (2) and mixed with the carotene fraction obtained from corn leaves. When this mixture was chromatographed on magnesia and alumina columns, one band was formed on the magnesia column and two bands on the alumina column. Thus it appears that, in the corn leaves studied, the carotene fraction consisted only of β-carotene and neo-β-carotene. Alpha-carotene was assumed not to be present since repeated chromatography of the carotene fraction on magnesia, an adsorbent on which alpha and beta carotene are easily separated, revealed only one band.

Quantitative spectrophotometric determinations for the carotenoid pigments were made immediately following the isolation of each pigment. A model DU Beckman spectrophotometer, supplied with cells having a light path of 1 cm, was used to determine the absorbance of the pigment solutions. The isolated carotenes were made to a volume of 10 ml with hexane and analyzed as a binary mixture by the method of Beadle and Zscheile (2). The other carotenoids were each made to a volume of 10 ml with 95 % ethanol and the absorbance of each carotenol solution was determined at its respective maximum. Strain's (11) specific absorption coefficients for carotenoids isolated from Avena seedlings were used to convert the absorbance values to pigment concentration. The observed maxima were: lutein 4460 Å, flavoxanthin 4500 Å, and violaxanthin 4420 Å. Strain's (11) specific absorption coefficients at these maxima are: lutein 245.5; flavoxanthin 234.5, and violaxanthin 231.7. Although neoxanthin was isolated, it was difficult to elute from the column and, therefore, was not determined. It was usually present in about the same amount as violaxanthin b.

The analytical methods used to extract and separate the carotenoid pigments were evaluated by determining the percentage recovery of pigments added to leaf samples before extraction. The percentage recoveries of the added pigments were: carotenes 96 %, lutein 95 %, flavoxanthin 92 % and violaxanthin 93 %.

A modification of the method of Zscheile and Comar (15) was used to extract the chlorophyll pigments from the leaf material. The sample to be analyzed was ground in a mortar containing sand, 0.1 gm of calcium carbonate, and 20 ml of a mixture of petroleum ether and methanol (75 to 90 % petroleum ether). The resulting brei was filtered through a medium sintered glass filter and the residue washed with methanol until it was white. The filtrate was transferred to a separatory funnel and the methanol washed out with water. The remaining petroleum ether solution contained the cholorphylls. The entire extraction was carried out in diffuse light of 1 fc in-

tensity. The chlorophylls were readily separated from the carotenoids and resolved into chlorophyll a and chlorophyll b by chromatographing the petroleum ether solution on a starch column. Chromatographic tubes similar to those used by Zechmeister and Cholnoky (14) were employed in the preparation of the columns and the chromatograms were developed with petroleum ether containing 4 % ethyl ether. After the chlorophylls were separated, the adsorbent was removed from the tube and the two chlorophyll bands cut out with a sharp scalpel. Each pigment was eluted with ethyl ether, the eluate collected in a flask and made to a volume of either 10 or 25 ml. The absorption spectra of the pigments eluted from the two bands were found to correspond very closely to those for chlorophyll a, obtained from corn leaves, and chlorophyll b, obtained from barley by Zscheile and Comar (15). The main absorption peaks for chlorophyll a were located at 4300 Å and 6650 Å; for chlorophyll b at 4530 Å and 6500 Å.

Quantitative spectrophotometric determinations for the chlorophyll pigments were made immediately following the isolation of each pigment. The concentrations of the chlorophyll pigments were calculated from the absorbance and Zscheile and Comar's (15) specific absorption coefficients. The absorbance of the chlorophyll a solution was determined at 4300 Å and that of the chlorophyll b solution at 4530 Å. Zscheile and Comar's (15) specific absorption coefficient for chlorophyll a at 4300 Å is 147 and for chlorophyll b at 4530 Å is 173.

Experimental

The changes in the carotenoid pigment content of corn seedlings grown in darkness at 25° C were determined as a function of time. Daily samples of the second seedling leaf were analyzed for carotenoid pigments from the 6th through the 14th day after planting. Three samples, each consisting of 5 leaf punches having a total fresh weight of about 0.27 gm, were used in each analysis. The data from these analyses are shown in figure 1.

In a second experiment, seedlings were grown in the dark at 25° C for 6 days after the time of planting the seed. Following the 6-day dark period, the seedlings were exposed to continuous light of an average intensity of 700 fc. At 0 and 6 hours after exposure to light and thereafter at daily intervals, 3 leaf samples were collected and analyzed for plastid pigments. Duplicate collections were made. From the first collection chlorophyll concentrations were determined and from the second carotenoid concentrations. The results of the carotenoid analyses are shown in figure 2 and those of the chlorophyll analyses in figure 3.

Results

The data in figure 1 show that lutein is the most abundant carotenoid pigment in dark-grown corn seedlings and that violaxanthin, flavoxanthin, β-carotene and neo-β-carotene are present in lesser amounts in the order listed. In the dark-grown seedlings the

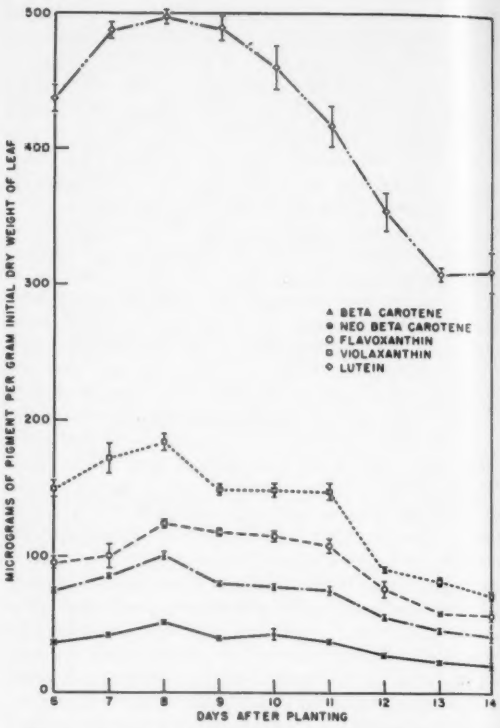

Fig. 1. Carotenoid content of dark-grown corn seedlings as a function of time after planting of seed. Corn seedlings were germinated and grown in darkness and the concentrations of the carotenoid pigments in the second leaf were determined daily from the 6th through the 14th day after planting the seed. The means and standard errors of the means are plotted.

concentration of each carotenoid pigment increased slightly during the first 3 days of analyses. Thereafter the concentrations decreased, until the final two days of the analyses. On the final two days of analyses the distal portion of the second leaf was brown, indicating the leaf had begun to deteriorate.

Since all of the pigments followed similar courses of accumulation and loss, the relative concentrations of pigments in the dark-grown seedlings did not change. However, this was not the case for the pigments in dark-grown corn seedlings exposed to continuous light. As the data in figure 2 show, the exposure of dark-grown corn seedlings to light resulted in an immediate decrease in the concentrations of flavoxanthin, violaxanthin and lutein and a rapid increase in the concentrations of β-carotene and neo-β-carotene. The decreases in the concentrations of violaxanthin and lutein were virtually complete by the end of the first day, while the decrease in flavoxanthin extended over a period of 3 days. The increase in the concentration of β-carotene continued for a period of 4 days, while the accumulation of neo-β-carotene was

complete within 3 days. Initially the concentrations of pigments in the dark-grown seedlings in order of decreasing amounts were: lutein, violaxanthin, flavoxanthin, β-carotene and neo-β-carotene. However, exposure to light altered the order to: β-carotene and lutein, neo-β-carotene, flavoxanthin and violaxanthin.

From the data in figure 3 it is evident that the exposure of dark-grown seedlings to light resulted in the accumulation of chlorophyll a and chlorophyll b. The accumulation of chlorophyll a was very rapid and complete within 4 days, while that of chlorophyll b was gradual, extending over a period of 5 days. At the end of the first 6 hours of exposure to light, the ratio of chlorophyll a to chlorophyll b was 37 : 1. Thereafter the ratio decreased and at the end of the fourth day was 6 : 1, a ratio close to that found in non-starch forming monocotyledons (10).

Discussion

While much information can be obtained about the carotenoids by studying them as a group, it is important that the individual components also be considered. The data in the present study indicate that an environmental change can affect each component of the carotenoids in a different way or to a different degree. Thus it is apparent that caution should be used in interpreting data obtained from analyzing the carotenoids as a group.

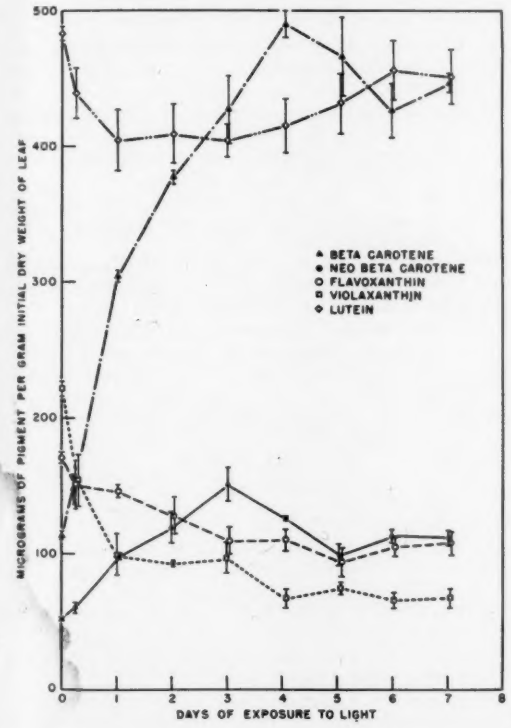
MICROGRAMS OF PIGMENT PER GRAM INITIAL DRY WEIGHT OF LEAF
DAYS OF EXPOSURE TO LIGHT
▲ BETA CAROTENE
● NEO BETA CAROTENE
○ FLAVOXANTHIN
□ VIOLAXANTHIN
◇ LUTEIN

Fig. 2. Carotenoid content of corn seedlings as a function of days of exposure to light. Corn seedlings were germinated and grown in the dark. Six days after planting the seedlings were exposed to continuous light and the concentrations of carotenoid pigments in the second leaf were determined at intervals over a 7-day period. The means and standard errors of the means are plotted.

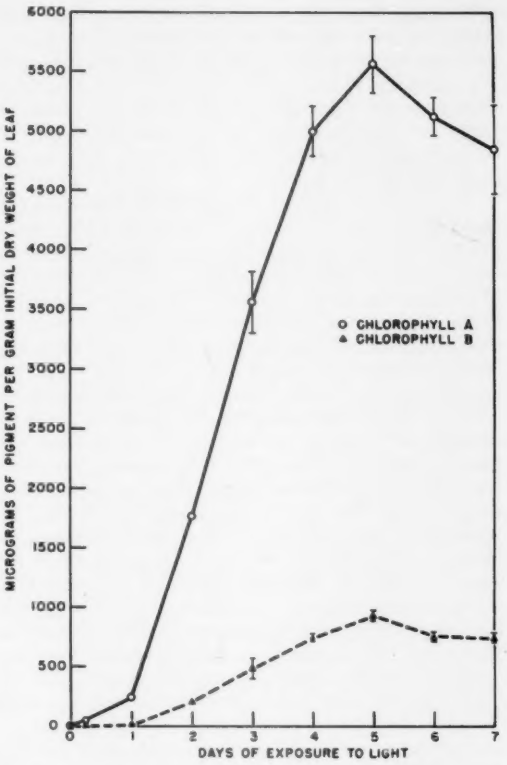

Fig. 3. Chlorophyll content of corn seedlings as a function of days of exposure to light. Corn seedlings were germinated and grown in the dark. Six days after the time of planting, the seedlings were exposed to continuous light and the concentrations of chlorophyll a and chlorophyll b were determined at intervals over a 7-day period. The means and standard errors of the means are plotted. The standard error is not shown when the value is less than the vertical dimension of the symbol used to indicate the mean value.

Frank (3, 4) has observed that the exposure of dark-grown seedlings to light results in a reduction in total carotenoids and the formation of chlorophyll. She has suggested that this reduction in carotenoids is due to the diversion of carotenoid precursors into phytol groups. However, in the present study exposure of dark-grown seedlings to light did not affect all of the carotenoids in the same way. The concentration of the carotenes increased while the concentration of the carotenols (flavoxanthin, lutein and violaxanthin) decreased. Therefore, if a reduction in carote-

noids concomitant with an increase in chlorophyll is considered to be the result of a diversion of carotenoid precursors to phytol groups, it appears that only the carotenol (flavoxanthin, lutein and violaxanthin) precursors are diverted into phytol groups. Thus it might be more profitable to study the relationship of the chlorophylls to the carotenols rather than to the total carotenoids (3, 4, 5).

In a study designed to determine the nature of the increase in carotenes induced by exposure of dark-grown plants to light, Bandurski (1) isolated the carotenes from the other carotenoids. From his work on the isolated carotene fraction he concluded that the rapid synthesis of carotenes is dependent upon the availability of photosynthetic products. On the other hand, Withrow's (13) data are at variance with this conclusion since they indicate that photosynthetic products are not necessary for the production of large amounts of carotene. In experiments with bean and corn seedlings he found that large amounts of carotene accumulated when plants were exposed to low irradiance at 725 mμ, a wave length at which no chlorophyll was formed and therefore no photosynthesis possible. However, an examination of Withrow's method of extracting carotenes indicates that his procedure extracts carotenols as well as carotenes. Therefore, his spectrophotometric method of analysis measured the carotenoid concentration (carotenols plus carotenes) rather than carotene concentration. Thus the "carotene" content of his dark-grown corn seedlings is 4 times that of our dark-grown plants. Since Withrow actually measured carotenoid content, his data does not necessarily show that large amounts of carotenes are produced in the absence of photosynthetic products.

The absence of α-carotene from corn leaves agrees with the finding of MacKinney (6), but it does not agree with that of Moster (8). The conditions under which the plants were grown may be responsible for the different results. The environment in which plants are grown is undoubtedly responsible for the absence of zeaxanthin from corn leaves. In the present study seedlings were grown under a light intensity of 700 fc and at a temperature of 25° C and no zeaxanthin was detected. However, the absence of zeaxanthin was not unexpected since Moster (7) has shown that each of these conditions is extremely unfavorable for the accumulation of zeaxanthin.

It should be noted that the ratios of carotenoids we have found in light-grown corn seedlings, as contrasted to dark-grown corn seedlings, are similar to those reported for other monocotyledonous seedlings such as barley (12).

Summary

A strain of maize (single cross L 289 × L 205) was grown under a controlled environment of light and temperature. The changes in the concentrations of the major carotenoid pigments and in the concentration of chlorophylls have been followed for a 7-day period, using only the second seedling leaf for the pigment analyses. Beta carotene, neo-β-carotene, lutein, flavoxanthin, violaxanthin, chlorophyll a and chlorophyll b were isolated as separate pigments. In both dark-grown and light-grown material, neoxanthin was also identified and was present in amounts comparable to that of violaxanthin. However, no α-carotene or zeaxanthin was found in the preparations.

In dark-grown plants lutein was found to be the major leaf carotenoid with violaxanthin, flavoxanthin, β-carotene and neo-β-carotene present in lesser amounts in the order listed. In these plants the concentration of each carotenoid pigment increased during the first 3 days of observation and thereafter it decreased. On the other hand, within 6 hours following the illumination of dark-grown seedlings, the concentrations of flavoxanthin, lutein and violaxanthin decreased and the concentrations of β-carotene and neo-β-carotene increased. After 4 days of exposure to light β-carotene and lutein were the most abundant carotenoid pigments; while neo-β-carotene, flavoxanthin and violaxanthin were present in lesser amounts.

Literature Cited

1. Bandurski, R. S. Synthesis of carotenoid pigments in detached bean leaves. Bot. Gaz. 111: 95–109. 1949.
2. Beadle, B. W. and Zscheile, F. P. Studies on carotenoids. II. The isomerization of beta carotene and its relation to carotene analyses. Jour. Biol. Chem. 144: 21–33. 1942.
3. Frank, S. The relation between carotenoid and chlorophyll pigments in Avena coleoptiles. Arch. Biochem. 30: 52–61. 1951.
4. Frank, S. and Kenney, A. L. Destruction of chlorophyll and carotenoids in corn seedlings. Federation Proc. 13: 47–48. 1954.
5. Frank, S. and Kenney, A. L. Chlorophyll and carotenoid destruction in the absence of light in seedlings of *Zea Mays* L. Plant Physiol. 30: 413–418. 1955.
6. MacKinney, G. Leaf carotenes. Jour. Biol. Chem. 111: 75–84. 1935.
7. Moster, J. B. and Quackenbush, F. W. The effect of temperature and light on the carotenoids of seedlings grown from three corn hybrids. Arch. Biochem. Biophys. 38: 297–303. 1952.
8. Moster, J. B., Quackenbush, F. W. and Porter, J. W. The carotenoids of corn seedlings. Arch. Biochem. Biophys. 38: 287–297. 1952.
9. Phinney, B. O. and Kay, R. E. Interaction of environment and genotype in the expression of a virescent gene, pale-yellow-1, of maize. Hilgardia 23: 185–196. 1954.
10. Rabinowitch, E. Photosynthesis and Related Processes, vol. I. Pp. 1–599. Interscience Publishers, Inc., New York 1945.
11. Strain, H. H. Leaf Xanthophylls. Carnegie Inst. Wash. Publ. No. 490. 1938.
12. Strain, H. H. Formation of carotenoids and chlorophylls in etiolated barley seedlings exposed to red light. Plant Physiol. 13: 413–418. 1938.
13. Withrow, R. B., Klein, W. H., Price, L. and Elstad, V. Influence of visible and near infrared radiant energy on organ development and pig-

ment synthesis in bean and corn. Plant Physiol. 28: 1–14. 1953.
14. ZECHMEISTER, L. and CHOLNOKY, L. Principles and Practice of Chromatography. Pp. 1–362. John Wiley and Sons, New York 1948.
15. ZSCHEILE, F. P. and COMAR, C. L. Influence of preparative procedure on the purity of chlorophyll components as shown by absorption spectra. Bot. Gaz. 102: 463–481. 1941.

STUDIES ON 3-INDOLEACETIC ACID METABOLISM. II. SOME PRODUCTS OF THE METABOLISM OF EXOGENOUS INDOLEACETIC ACID IN PLANT TISSUES[1,2]

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It has long been known that indoleacetic acid (IAA) is taken up by plants and translocated to various tissues, causing characteristic modifications in the pattern of growth. However the fate of IAA in vivo has received little attention. This is surprising in that IAA and its derivatives are virtually the only materials of known chemical constitution which both exhibit the formative effects of plant growth substances and occur naturally in plants. The fact that the concentrations of endogenous IAA are extremely low is no doubt in part responsible for the neglect of an important field of research. The authors of this paper have attempted to overcome the difficulty by supplying much larger amounts from an ambient solution. They are quite aware, however, that the reactions associated with relatively high concentrations of applied IAA may differ significantly from metabolic reactions as they occur in nature.

Exogenous IAA participates in several biological reactions. Plants are known to contain IAA oxidizing enzymes and part of the IAA administered to plants presumably undergoes oxidative degradation to products which are no longer growth active (8). Hemberg (3) has noted a rise in "bound auxin" in IAA treated corn kernels. Siegel and Galston (6) have reported that IAA administered to excised pea roots becomes attached to proteins. On the other hand we have recently found that IAA administered to pea stems is conjugated with aspartic acid (1). The present communication constitutes a report on the extention of our investigations to other plant species.

MATERIALS AND METHODS

A survey was carried out using 12 plant species belonging to 8 families. The following tissues were investigated: etiolated coleoptiles of oats, corn and barley all harvested just before the emergence of the primary leaf; etiolated epicotyls of peas 7 days old; etiolated hypocotyls of sunflower, cucumber and buckwheat, 7, 4, and 4 days old respectively; etiolated potato sprouts 3 to 5 inches long; stems and petioles of greenhouse grown pea, tomato, and cabbage plants. Immediately after harvesting, the tissue was weighed and cut into pieces aproximately 2 inches long. To each 100 gms of tissue were added 2 liters of M/60 sodium dihydrogen phosphate with or without 60 mg of IAA.[3] The floating tissue was incubated at 24° C for 24 hours in the dark with gentle shaking. After this period the tissue was removed, thoroughly washed, and frozen at −8° until it could be conveniently extracted. The residual IAA in the ambient solutions was measured by the acid-ferric chloride (Salkowski) method (8) and in some cases the solutions were ether extracted and chromatographically analyzed for other Salkowski-positive substances.

To each 150 gm of frozen tissue was added 65 ml of 0.3 *N* sodium bicarbonate solution. The tissue was then ground in a Waring blendor and filtered through cheese cloth. The resulting brei was saturated with ammonium sulfate, infusorial earth was added and the precipitated proteins were removed by filtration on a Buchner funnel. (The unwashed precipitate from pea contained from 10 to 20 % of the Salkowski-positive material. However, when the precipitate was thoroughly washed by redissolving it in water and reprecipitating it with ammonium sulphate, practically all the Salkowski-positive material was in solution. Since the washings contained the same substances as the filtrate and in about the same proportions only the first filtrates were regularly investigated in this survey.) The filtrate (pH about 7.5) was extracted once with 100 ml and twice with 50 ml of peroxide-free ether, acidified to pH 2.5 with phosphoric acid and extracted with ether as before. Then the acidified filtrate was extracted with 100, 50 and 20 ml portions of 1-butanol. The aqueous residue was by this time Salkowski-negative. The acidic extracts were made slightly alkaline either with sodium bicarbonate solution or with dilute ammonia and taken to dryness, the

[1] Received October 11, 1955.
[2] Contribution No. 62, Canada Department of Agriculture, Science Service Laboratory, University Sub Post Office, London, Ontario, Canada.

[3] Some samples of indoleacetic acid from commercial sources are very impure and contain appreciable amounts of indoleacetamide and other Salkowski reactive substances. The following purification procedure, however, has been found to be effective: Commercial IAA is dissolved in water (about 100 ml/gm) containing an excess of sodium bicarbonate. The aqueous solution is extracted several times with 1-butanol, then several times with ether. The dissolved ether is removed by aeration and the solution is slowly acidified to pH 3.0 with phosphoric acid. About 70 % of the original IAA separates on standing as white, flake-like crystals.

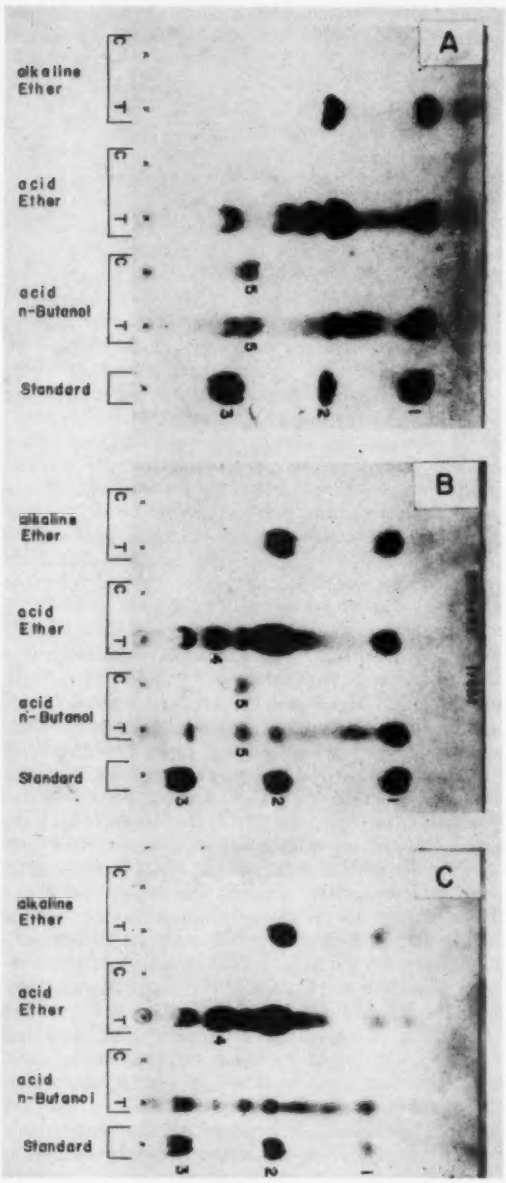

FIG. 1. Typical chromatograms of extracts of IAA treated (T) and untreated (C) plant tissues (sunflower hypocotyls) obtained by successive alkaline-ether, acid-ether and acid-butanol extractions. East spot represents the extract from 3 gm fresh wt of tissues. Chromatogram (A) was developed with an isopropanol–ammonium acetate–acetic acid solvent and sprayed with the Ehrlich reagent (*p*-dimethylaminobenzaldehyde–HCl). Chromatograph (B) was developed with an isopropanol-ammonia solvent and sprayed with the Ehrlich reagent. Chromatogram (C) was developed with the same isopropanol-ammonia solvent but sprayed with the Salkowski reagent (ferric chloride–perchloric acid). Spot 1 is synthetic indoleacetamide, spot 2 is indoleacetic acid and spot 3 is synthetic indoleacetylaspartic acid, each 0.05 micromoles. Spot 4 is an unknown acidic substance described in the text, and spot 5 is tryptophan.

ether extracts in an air stream and the butanol extracts by distillation at reduced pressure. The solvent-free residues were taken up in 1.0 ml of dilute aqueous ammonia or bicarbonate solution. An aliquot (20 μl) was immediately chromatographed on paper and the remainder was stored at $-8°$ C.

The concentrated extracts were chromatographed on 15″ × 15″ sheets of Whatman #1 filter paper for 16 hours at 24° C, using the ascending solvent techniques. The principal partitioning solvents were:

(A) 80 parts of 2-propanol to 20 parts of 8 *N* ammonium hydroxide solution (v/v).

(B) 80 parts of 2-propanol containing 1.25 % acetic acid to 20 parts of a 20 % aqueous solution of ammonium acetate (v/v).

Equivalent extracts from the untreated and the IAA treated tissues were chromatographed as adjacent spots. The dried chromatograms were sprayed either with a modified Salkowski reagent (0.05 M $FeCl_3$ in 35 % $HClO_4$) or with the Ehrlich reagent (1 % *p*-dimethylaminobenzaldehyde in 50 % alcohol–50 % concentrated HCl). See figure 1. In some cases regions on unsprayed chromatograms corresponding to the colored regions on sprayed chromatograms were eluted with water or dilute aqueous ammonia and rechromatographed. The resulting purified material was again eluted, its ultraviolet absorption spectrum determined and its hydrolysis products tested for IAA, amino acids and other substances. When the survey was completed the frozen acid-ether and butanol extracts were thawed and chromatographed simultaneously (see fig 2).

RESULTS

The Salkowski and Ehrlich-reactive IAA derivatives were similar in the various plants grown under different conditions. Figure 1 shows typical chromatograms of successive extracts, in this case of sunflower hypocotyls. Similar chromatograms were made with extracts from each species. Tissues which had not been treated with IAA (C) were devoid of Salkowski-reactive material and the only Ehrlich-positive substance, which occurred alike in treated and untreated plants, was tryptophan (spot 5). This was true of all 12 plants examined. For purposes of comparison the extracts of the 12 plant species were chromatographed together to form the composite picture shown in figure 2; space limitation did not permit inclusion of the controls. There were 4 major Salkowski-reactive substances and only these have been studied in detail. A description of the numerous minor components will entail a more intensive study of the tissue extracts.

3-INDOLEACETAMIDE: All the extracts of IAA

treated plants, with the possible exception of cucumber, contained neutral rapid-moving substances chromatographically identical with 3-indoleacetamide (spot 1, figs 1 and 2). These materials, like synthetic indoleacetamide, gave the purple color typical of IAA with the Ehrlich reagent and a slowly developing weak color with the Salkowski reagent. A sample from corn coleoptiles had the ultraviolet absorption spectrum of indole compounds with maxima at 280 and 288 mμ. It yielded IAA and ammonia (as determined by the Nessler reaction) in approximately equimolar amounts on hydrolysis. Apparently indoleacetamide occurs in particularly large amounts in IAA treated grasses (fig 2).

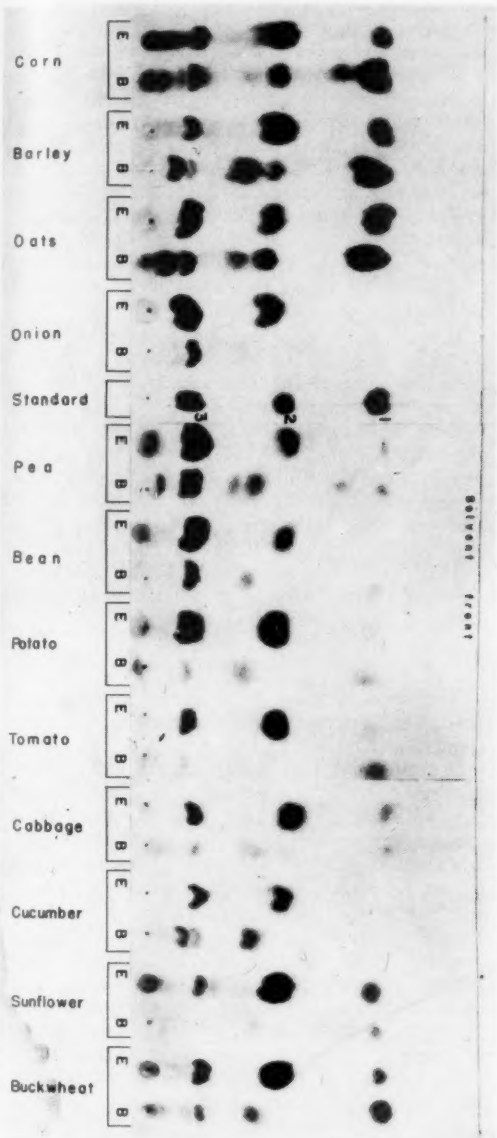

FIG. 2. Chromatograms of acid-ether (E) and acid-butanol (B) extracts from the IAA treated tissues of 12 plant species. Each spot represents the extract from 3 gm fresh wt of tissue. The chromatograms were developed with an isopropanol-ammonia solvent and sprayed with the Ehrlich reagent. Spot 1 is synthetic indoleacetamide, spot 2 is indoleacetic acid, and spot 3 is synthetic indoleacetylaspartic acid, each 0.05 micromoles.

3-INDOLEACETIC ACID: Each extract of IAA treated plants contained an acidic substance chromatographically identical with IAA (spot 2, figs 1 and 2).

3-INDOLEACETYLASPARTIC ACID: The acid-ether and butanol extracts of all the IAA treated plants contained a slow-moving acidic substance chromatographically identical with the indoleacetylaspartic acid (spot 3, figs 1 and 2) previously reported in pea (1). The earlier report stressed the facts that indoleacetylaspartic acid was not extracted by ether and that it did not occur in barley. Both of these assertions must be qualified. Because of its increased acid strength, presumably due to the presence of an acylated amino group adjacent to one of its carboxyls, indoleacetylaspartic acid is only extracted by ether from strongly acidic solutions (about pH 3.0) and thus can be separated easily from IAA. Moreover indoleacetylaspartic acid or some very similar substance does occur in barley but in such small amounts that its presence was missed until highly concentrated extracts were examined (fig 2).

AN UNIDENTIFIED ACIDIC SUBSTANCE: Another acidic material occurred in the acid-ether extracts of many IAA treated plants, particularly in the extracts of sunflower (spot 4, fig 1 C), tomato, bean, cucumber and pea. This substance appeared approximately midway between IAA and indoleacetylaspartic acid on chromatograms developed with the 2-propanol-ammonia solvent. There was some evidence that two compounds may have been involved since the R_f value of the material from sunflower was considerably higher than that of the comparable material from cucumber. The substance (or substances) gave an immediate bright pink or scarlet color with either acid–ferric chloride (Salkowski) or acid-nitrite sprays and could be readily distinguished from IAA by the speed of the color development. It gave a very weak, atypical (grey) color with the Ehrlich reagent. Since none of it was extracted by ether from weakly basic or neutral solutions, it is probably a stronger acid or its salts are less soluble in ether than is the case with IAA. The undissociated form, however, was almost quantitatively removed by ether from acidified aqueous solutions. A sample obtained from sunflower hypocotyls was unstable to boiling with either acid or base but no IAA or other Salkowski-positive material could be found after the treatment. The substance could not be detected among the products of IAA oxidation when the oxidation was brought about enzymatically by oxygen or non-enzymatically by perbenzoic acid, hypochlorite or permanganate.

None of the compounds described above appeared in the ambient solution during the incubation of the plant tissues and therefore it is unlikely that the metabolism of inadvertently introduced bacteria could have contributed any of these derivatives.

Relatively little of the IAA lost from solution could be found in the tissue extracts by means of the color tests used. Table I shows the quantitative discrepancy between the disappearance of IAA and the accumulation of extractable Salkowski-reactive material in the plants. An attempt to identify the missing unreactive substances has been made, without success to date. Alkaline hydrolysis of the tissue proteins did not release IAA or recognizable degradation products. Sprays containing nitrous acid, diazotized sulphanilic acid, diazotized *p*-nitroaniline, and 2,4-dinitrophenylhydrazine did not reveal significant amounts of new substances on the chromatograms of tissue extracts. Similarly, unspecific oxidants such as ferric chloride–ferricyanide mixtures detected only the spots already observed with the aid of the conventional indole reagents. However, the precipitated proteins, the aqueous residue after butanol extraction and the ambient solution have not yet been examined for Salkowski- and Ehrlich-negative IAA derivatives. It would seem that the investigation of these more elusive substances must await the development of improved methods for their detection.

TABLE I

THE CONVERSION OF IAA BY PLANT TISSUES INTO SUBSTANCES LESS CHROMOGENIC WITH THE SALKOWSKI REAGENT

TISSUE	SALKOWSKI COLOR ** DEVELOPED WITH COMBINED TISSUE EXTRACTS AND EXPRESSED AS AMOUNT OF IAA REQUIRED TO GIVE THE SAME COLOR	LOSS OF IAA FROM THE AMBIENT SOLUTION	DECREASE IN SALKOWSKI REACTION OF THE AMBIENT SOLUTION ACCOUNTED FOR BY THE REACTION OF THE TISSUE EXTRACTS
	mg	*mg*	*%*
Corn	6	52	11.5
Barley	6	64	9.5
Oats *	2.5	54	4.5
Onion	5	50	10
Pea	12.5	70	18
Bean	7	20	3.5
Tomato	2.5	15	16.5
Potato	4	34	12
Cabbage	2.5	0 (?)	?
Cucumber	1	21	5.0
Sunflower	3	15	20
Buckwheat	4	16	25

The data refer to 150 gm fresh wt of tissue incubated for 24 hrs at 24° C in 3 liters of M/60 NaH_2PO_4 solution containing 90 mg IAA.

* 75 gm fresh wt.

** IAA in the ambient solution and Salkowski reactive materials in the tissue extracts were determined by the method of Tang and Bonner (8) except that the readings were taken 2 hrs after the addition of the Salkowski reagent (1).

DISCUSSION

It is not known which compounds derived from exogenous IAA are also formed from endogenous IAA and consequently the pertinence of our observations to the problems of the normal metabolism of plant growth hormones has not been established. Other workers have reported a number of Salkowski-reactive substances from plant extracts. Among these were indoleacetic acid (2), indolepyruvic acid (7), the ethyl ester of indoleacetic acid (5), and indoleacetonitrile (4). Indoleacetylaspartic acid and indoleacetamide have not yet been reported as naturally occurring and judging by the published information there is no reason to believe that they have been encountered without being recognized.

In spite of the large number of Salkowski-positive substances found in the treated tissues (at least 7 in sunflower) most of the IAA lost from the ambient solution was converted into Salkowski- and Ehrlich-negative substances, or at least, into substances which give very much weaker colors with both these reagents. Since the Ehrlich reagent as used in the experiments reacts fairly generally with 3-substituted indoles, it is probable that the degradation involves an early ring cleavage. Radioactive indoleacetic acid with C^{14} in the aromatic nucleus is being synthesized and will be employed in future studies to locate these less easily recognized substances.

SUMMARY

Tissues from twelve plant species have been incubated with indoleacetic acid. Extracts of these tissues have been examined for IAA derivatives.

A large number of substances which give characteristic colors with the Salkowski or Ehrlich reagents were found in all the IAA treated tissues. Those occurring most generally and in largest amounts were, on the basis of chromatographic behavior and hydrolysis products, indoleacetamide, unchanged indoleacetic acid, and indoleacetylaspartic acid. An unknown acidic compound which reacts with the Salkowski reagent but not with the Ehrlich reagent occurred in several plants.

No convincing qualitative differences in the products of IAA metabolism were noted among the plants investigated. However, indoleacetamide predominated in the grasses and indoleacetylaspartic acid predominated in the legumes and in onion.

Most of the indoleacetic acid which disappeared could not be accounted for by substances reacting with either of the reagents.

LITERATURE CITED

1. ANDREAE, W. A. and GOOD, NORMAN E. The formation of indoleacetylaspartic acid in pea seedlings. Plant Physiol. 30: 380–382. 1955.
2. BENNET-CLARKE, T. A., TAMBIAH, M. S. and KEFFORD, N. P. Estimation of plant growth substances by

partition chromatography. Nature 169: 452–453. 1952.
3. HEMBERG, T. Studies on the balance between free and bound auxin in germinating maize. Physiol. Plantarum 8: 418–432. 1955.
4. JONES, E. R. H., HERBERT, H. B., SMITH, G. F. and BENTLEY, J. A. 3-Indolylacetonitrile: a naturally occurring plant growth hormone. Nature 169: 485–487. 1953.
5. REDEMANN, S. T., WITTWER, S. H. and SELL, H. M. The fruit-setting factor from the ethanol extracts of immature corn kernels. Arch. Biochem. Biophys. 32: 80–84. 1951.
6. SIEGEL, M. G. and GALSTON, A. W. Experimental coupling of indoleacetic acid to pea root protein in vivo and in vitro. Proc. Nat. Acad. Sci., U. S. 39: 1111–1118. 1953.
7. STOWE, B. B. and THIMANN, K. V. Indolepyruvic acid in maize. Nature 172: 764. 1953.
8. TANG, Y. W. and BONNER, J. The enzymatic inactivation of indoleacetic acid. I. Some characteristics of the enzyme contained in pea seedlings. Arch. Biochem. 13: 11–25. 1947.

STUDIES ON 3-INDOLEACETIC ACID METABOLISM. III. THE UPTAKE OF 3-INDOLEACETIC ACID BY PEA EPICOTYLS AND ITS CONVERSION TO 3-INDOLEACETYLASPARTIC ACID[1,2]

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In two earlier publications (2, 5) it was reported that 3-indoleacetic acid (IAA) administered to plant tissues is taken up and converted in part to derivatives which still give positive Salkowski (acid-$FeCl_3$) reactions. The two major derivatives which react with this reagent were identified as 3-indoleacetylaspartic acid and 3-indoleacetamide. Indoleacetamide predominates in grasses while indoleacetylaspartic acid is by far the most abundant Salkowski reactive derivative accumulating in legumes.

The present paper deals with factors affecting the uptake of IAA and its conversion to Salkowski reactive derivatives in pea epicotyls. Paper chromatographic studies have shown that indoleacetylaspartic acid represents about 90 % of the Salkowski reactive IAA derivatives and consequently the present study is essentially one of the formation of indoleacetylaspartic acid. In this investigation IAA and indoleacetylaspartic acid were estimated independently by extracting lyophilized tissues with ether; indoleacetylaspartic acid, which is insoluble in ether under these conditions was then extracted with bicarbonate solution, leaving a Salkowski unreactive residue. For the sake of convenience, the Salkowski positive materials in the ether and bicarbonate fractions will be referred to as "IAA" and "indoleacetylaspartic acid" respectively although in fact both fractions also contained small amounts of unknown IAA derivatives. The procedure used was not sensitive enough to detect the IAA and other Salkowski positive materials which occur naturally in the tissues.

MATERIALS AND METHODS

Pea epicotyl sections were obtained from Alaska peas grown on vermiculite in the dark at 27° C (80° F) and 80 % relative humidity, exposed to red light for 2 hours on the 6th day and harvested on the seventh. The epicotyls, with their terminal buds removed, were cut into approximately 2-inch sections and 10-gram lots were floated on 500 ml of M/60 NaH_2PO_4 solution containing varying amounts of IAA. The pH of the ambient solution was about 4.6. The suspended epicotyls were shaken in the dark at room temperature for intervals of varying duration. Before and after incubation 1-ml aliquots were removed from the ambient solution and treated with 4 ml of the Salkowski reagent (0.001 M $FeCl_3$ in 14 N H_2SO_4). After 20 minutes the intensity of the pink color was determined photometrically as described by Tang and Bonner (9).

After incubation with IAA the tisues were removed from the solution and washed with water. In some experiments they were then immediately frozen and lyophilized; in other experiments the tissues were placed for various intervals of time on glass plates in an atmosphere saturated with water vapor in the dark at room temperature after which they were frozen and lyophilized.

In both cases the lyophilized epicotyls were weighed and ground in a Wiley mill to pass a 40-mesh sieve. Two-hundred-milligram aliquots of the dry powder were then extracted 4 times with 5 ml of water-saturated, peroxide-free ether. The ether extracts were combined, filtered, evaporated to dryness and taken up in 2 ml of 0.1 N $NaHCO_3$. To 1 ml of the concentrated extract was added 4 ml of the Salkowski reagent and the intensity of the color formed determined as described above. The powder, after ether extraction, was suspended in 4 ml of 0.1 N $NaHCO_3$ overnight at 5° C and then centrifuged. To 1 ml of the supernatant solution were added 4 ml of Salkowski reagent and the intensity of the mauve color characteristic of the Salkowski reaction with indoleacetylaspartic acid was determined except that the

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[2] Contribution No. 64, Science Service Laboratory, Canada Agriculture, University Sub Post Office, London, Ontario.

readings were taken after 2 hours, at which time the color had reached maximum intensity. It was found that the Salkowski color of indoleacetylaspartic acid after 2 hours was only 2/3 the intensity of an equimolar IAA solution read after 20 minutes. The colored indoleacetylaspartic acid–Salkowski complex obeys Beer's Law up to about 0.2 micromoles.

Experimental Results

The Recovery of Added IAA and Indoleacetylaspartic Acid from the Tissues: A preliminary study was carried out to determine the recovery of added IAA and indoleacetylaspartic acid from pea epicotyl material by the method described above. This was investigated in the following manner: to 200-mg samples of tissue powder was added 3 ml of dry acetone containing IAA and indoleacetylaspartic acid or both (table I). The samples were taken to dryness under vacuum at room temperature, extracted first with ether and afterwards with sodium bicarbonate solution and the extracts analyzed for IAA and indoleacetylaspartic acid. To study the interference by the plant extracts in the photometric analysis, ether and bicarbonate extracts of the lyophilized powder were similarly fortified with various concentrations of IAA and indoleacetylaspartic acid, and the intensity of the color after addition of the Salkowski reagent was determined (fig 1).

Table I indicates that IAA and indoleacetylaspartic acid added to tissue powder can be completely separated from each other by ether and bicarbonate extraction; no detectable IAA being found in the bicarbonate fraction or indoleacetylaspartic acid in the ether. The values obtained for IAA, however, were about 70 % of the amount of IAA added whether IAA

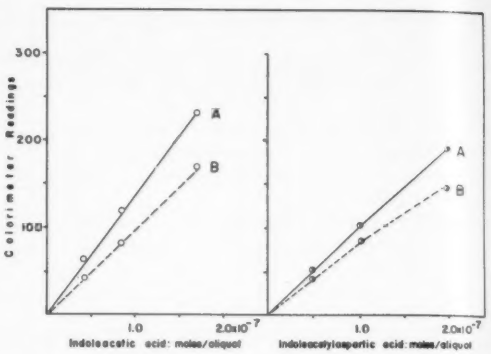

Fig. 1. The effect of pea epicotyl extracts on the color development of IAA and indoleacetylaspartic acid with the Salkowski reagent. To 1 ml of solution containing IAA and indoleacetylaspartic acid at various concentrations (Curves A) or to 1 ml of ether-bicarbonate or bicarbonate extracts of 200 mg pea powder fortified with IAA or indoleacetylaspartic acid (Curves B) was added 4 ml of Salkowski reagent and the resulting colors measured in a Klett-Summerson colorimeter at 540 mμ after 20 min (IAA) or 2 hrs (indoleacetylaspartic acid).

was added to the tissue powder before extraction or to the extract of the powder (fig 1). This indicates that the lower values found were caused by the extraction of substances which interfere with the Salkowski color development rather than by an incomplete extraction of the IAA. In the case of indoleacetylaspartic acid, the values found were about 60 % of the amount added when the lower concentrations were used, while with the highest concentration the recovery was only about 50 %. These low values appear to be due to two factors, one being the extraction from the powder of bicarbonate soluble materials (fig 1) which imparted a turbidity to the extract on the addition of the Salkowski reagent thus interfering with the photometric analysis. The other factor was incomplete recovery of the indoleacetylaspartic acid from the lyophilized powder; however, since two bicarbonate extractions failed to give better recoveries than one, only one extraction was used in routine experimental analysis. The IAA and indoleacetylaspartic acid concentrations in the following experiments are reported as those found; no corrections for incomplete recoveries were applied.

In subsequent experiments the tissues were immediately killed by briefly boiling in bicarbonate solution and ground in a Waring blendor. The brei was then saturated with ammonium sulfate and filtered. The values for the IAA extracted from the acidified filtrate (pH 4.5) by ether and for the indoleacetylaspartic acid remaining in the bicarbonate solution were about 30 % higher than those obtained by the procedure described above. However, data obtained by either method presented the same general picture of IAA metabolism.

Table I

Recovery of Added IAA and Indoleacetylaspartic Acid from Lyophilized Pea Epicotyl Powder by Ether and Bicarbonate Extraction

Addenda to 200 mg tissue powder moles × 10^7	Ether extraction		Bicarbonate extraction	
	Found moles × 10^7	Recovered %	Found moles × 10^7	Recovered %
IAA				
3.44	2.24	67	0	..
1.72	1.30	76	0	..
0.86	0.56	65	0	..
Indoleacetylaspartic acid				
8.4	0	..	4.02	48
4.2	0	..	2.61	62
2.1	0	..	1.54	73
IAA + Indoleacetylaspartic acid				
1.72 IAA + 4.2 indoleacetylaspartic acid	1.22	70	3.02	72

The results represent the means of 3 determinations.

THE EFFECT OF THE CONCENTRATION OF APPLIED IAA ON THE ACCUMULATION OF IAA AND INDOLEACETYLASPARTIC ACID IN THE TISSUES: Pea epicotyl sections were floated on 500 ml of M/60 NaH_2PO_4 solutions containing from 0 to 3.4×10^{-4} M IAA. After 24 hours gentle shaking in the dark, the tissues and ambient solutions were analyzed for IAA and indoleacetylaspartic acid. The results are shown in figure 2. The amount of IAA disappearing from solution depended upon the concentration of IAA applied; the loss increased most rapidly with increasing concentrations up to 0.75×10^{-4} M. When the concentration of applied IAA was below about 0.5×10^{-4} M very little or no free IAA could be detected in the tissues. Above this concentration, IAA did accumulate and above 1.0×10^{-4} M the content in the tissues rose rapidly with increasing concentrations of IAA in solution.

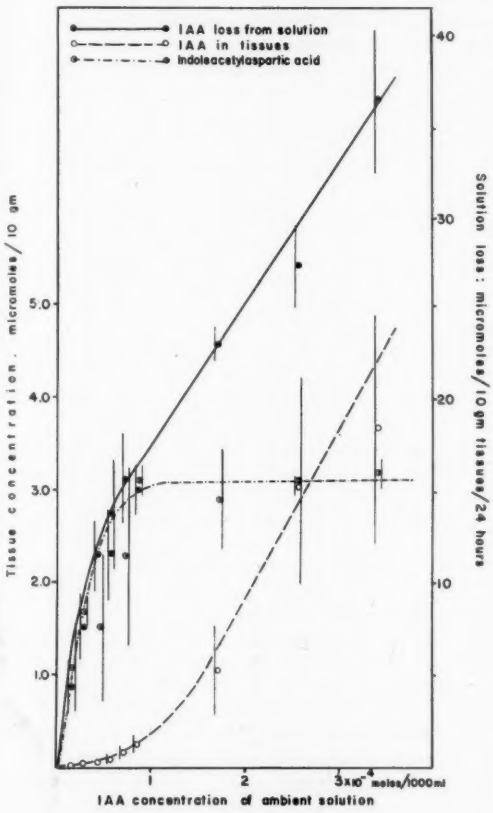
IAA loss from solution
IAA in tissues
Indoleacetylaspartic acid
Tissue concentration : micromoles / 10 gm
Solution loss : micromoles / 10 gm tissues / 24 hours
IAA concentration of ambient solution
3x10⁻⁴ moles/1000ml

FIG. 2. The effect of the concentration of applied IAA on its loss from solution, its accumulation in the tissues, and its conjugation with aspartic acid. Tissues were incubated in varying concentrations of IAA for 24 hrs as described in the text. The ambient solution and lyophilized tissues were then analyzed for IAA and indoleacetylaspartic acid. The vertical lines in the figure represent twice the standard error of the mean of 4 experiments.

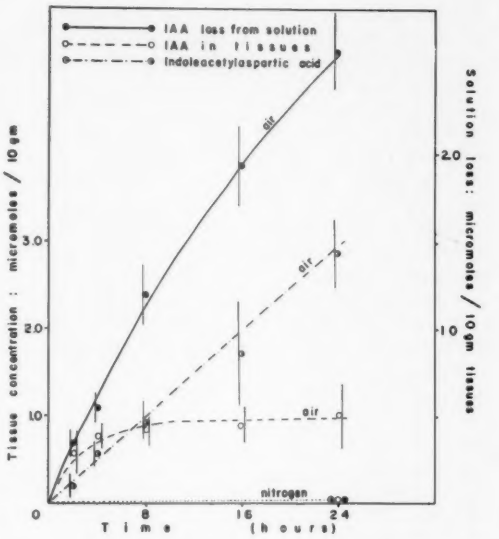

FIG. 3. The time course of the accumulation of IAA and indoleacetylaspartic acid in pea epicotyls. Tissues were incubated in 1.72×10^{-4} M IAA for 24 hrs in the presence or absence of oxygen. The solutions and tissues were analyzed at various intervals for IAA and indoleacetylaspartic acid. The vertical lines in the figure represent twice the standard error of the mean of 4 experiments.

When the tissues were bathed in solutions containing more than 1.5×10^{-4} M IAA, the concentration of IAA found in the tissues was about the same as that in solution. Indoleacetylaspartic acid, on the other hand, increased rapidly in the tissues with increasing concentrations of applied IAA up to 0.75×10^{-4} M. It then increased more slowly to a maximum value which was attained when the external IAA was about 1.0×10^{-4} M. At all concentrations the total IAA and indoleacetylaspartic acid in the tissues could account for only about 20 % of the IAA disappearing from solution. Hydrolysis of the ether and bicarbonate extracts with 6 *N* NaOH for 3 hours did not result in the appearance of IAA which could not be accounted for by the hydrolysis of Salkowski reactive derivatives. No IAA could be observed in the ether and bicarbonate extracted residues hydrolyzed under the same condition.

THE TIME COURSE OF THE ACCUMULATION OF IAA AND INDOLEACETYLASPARTIC ACID IN THE TISSUES: Pea epicotyl sections were floated on 500 ml of M/60 NaH_2PO_4 solutions containing 1.72×10^{-4} M IAA for periods of 2, 4, 8, 16 and 24 hours in air at room temperature. Parallel experiments were carried out under nitrogen. At the end of the incubation period, the ambient solutions and tissues were analyzed for IAA and indoleacetylaspartic acid. The results are shown in figure 3. The IAA content of the tissues rapidly rose during the first 4 hours and only slightly there-

after while the indoleacetylaspartic acid formation in the tissues proceeded at a constant rate of about 0.119 micromoles per 10 grams of tissue per hour. The loss of IAA from solution continued over the entire experimental period at a slightly decreasing rate which was again about 5 times the rate of the appearance of Salkowski reactive materials in the tissues. Increasing the area of cut surface by slitting the sections lengthwise had little effect on the loss of IAA from solution. In the absence of oxygen there was no loss of IAA from solution and no evidence of IAA and indoleacetylaspartic acid in the tissues.

THE CONVERSION OF INTERNAL IAA TO INDOLEACETYLASPARTIC ACID: Pea epicotyl sections were fortified with IAA by floating them on 500 ml of

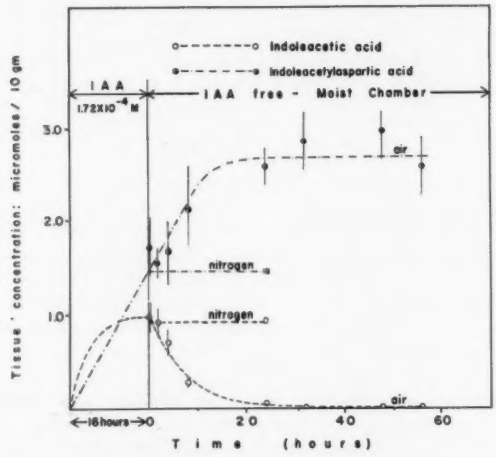

FIG. 4. The conversion of internal IAA to indoleacetylaspartic acid. Tissues were incubated in 500 ml of M/60 NaH_2PO_4 containing 1.72×10^{-4} M IAA for 16 hrs after which they were transferred to a moist chamber in presence or absence of air and analyzed after various intervals for IAA and indoleacetylaspartic acid. The vertical lines in the figure represent twice the standard error of the mean of 4 experiments.

M/60 NaH_2PO_4 solutions containing 1.72×10^{-4} M IAA for 16 hours after which they were washed and placed on glass plates in a chamber saturated with water vapor. The IAA and indoleacetylaspartic acid contents of tissue aliquots were determined at the end of the pretreatment and at intervals throughout the subsequent incubation in the moist chamber. The results are shown in figure 4. At the end of IAA incubation period, both IAA and indoleacetylaspartic acid were present in the tissues. After removal from the IAA solution and incubation in water saturated air the IAA content of the tissues declined until it could no longer be measured, while the Salkowski reactive IAA derivatives, which by chromatographic analysis were found to consist almost entirely of indoleacetylaspartic acid, appeared in the tissues in amounts approximately equal to the IAA lost. Once formed, the indoleacetylaspartic acid was stable over the experimental period.

In an atmosphere of nitrogen, there was no loss of IAA and no disappearance or formation of indoleacetylaspartic acid.

Essentially the same results were obtained if instead of first incubating for 16 hours with IAA, the tissues were infiltrated with solutions containing M/60 Na_2HPO_4 and IAA under vacuum for 30 minutes. In this case the pretreatment period was so short that no indoleacetylaspartic acid could be detected at the end. However, incubation in a moist chamber for 24 hours resulted again in an almost quantitative conversion of IAA into indoleacetylaspartic acid.

DISCUSSION

Because of the limitations of the analytical method the results on changes in the IAA and indoleacetylaspartic acid content of the epicotyl sections must be regarded as indicating trends rather than as representing absolute values. The absolute values would probably be about 30 % higher for IAA and from 40 to 50 % higher for indoleacetylaspartic acid.

In the course of these studies it was observed that the fate of IAA applied externally to tissues differed from that of IAA which was already in the tissues. When IAA was taken up from solution only about 20 % of the IAA which disappeared from solution could be accounted for as Salkowski reactive material in the tissues. These substances were chiefly IAA and indoleacetylaspartic acid. Their relative amounts depended upon the concentration of applied IAA, very little or no free IAA being detected when low concentrations were used. At higher concentrations, when the rate of indoleacetylaspartic acid formation became maximal, free IAA appeared in the tissues. On the other hand, when tissues fortified by a preliminary incubation with IAA were incubated in a moist chamber, nearly all the free IAA which disappeared from the tissues could be accounted for as indoleacetylaspartic acid.

How can these observations on the metabolism of IAA accumulated in the tissues be reconciled with the fate of IAA applied externally? It has been shown that the loss of IAA from solution is associated with the presence of the tissues. On removal of the tissues the disappearance of IAA ceased and therefore it is unlikely that IAA was destroyed by microorganisms or extracellular enzymes (5). It is possible that the major part of the IAA is converted during its passage into the cytoplasm into Salkowski unreactive substances, while only a fraction of the IAA actually enters the part of the cell where it is conjugated with aspartic acid. It is known that some metabolic activities are localized in discrete parts of the cell; some enzyme systems being associated with the cell surface, others with the particulate or the soluble constituents of the cell. A further possibility is that various tissues of the pea epicotyl react with IAA in different ways, certain tissues converting it to Salkowski unreactive derivatives as rapidly as it enters them, while

other tissues principally conjugate it with aspartic acid. Such different metabolic responses to IAA might well explain the different physiological responses of various tissues to this auxin.

In any case it seems that free IAA accumulates in tissues only at the sites concerned chiefly with its conjugation; otherwise subsequent incubation would result in destruction. Moreover free IAA accumulates only when the rate of entry exceeds the rates of conjugation.

The uptake of IAA by pea epicotyls under the conditions investigated is wholly dependent upon aerobic respiration, none being detected under nitrogen. This is in contrast to Sutter's (8) and Albaum's (1) conclusions, based on studies with Cucumis hypocotyls and Nitella cells respectively, that uptake is a simple diffusion process. It also differs from Reinhold's findings (6) on the uptake of IAA by carrot sections in which two processes, one physical and one metabolic, appear to operate. However the concentrations of IAA used by Reinhold (6) and by Albaum et al (1) were much greater than those used in the present work.

As yet little can be said regarding the metabolic activities involved in the conversion of IAA to Salkowski reactive and unreactive derivatives by the tissues. The mechanism of formation of indoleacetylaspartic acid appears to be linked to respiration as it ceases in absence of oxygen. It is possible that the reaction may require the intermediate formation of an indoleacetyl-coenzyme A thioester which then reacts with aspartic acid to give indoleacetylaspartic acid by a process analogous to the formation of hippuric acid (4).

Since there was no evidence of the formation of a Salkowski reactice protein complex of the type found by Siegel and Galston (7) in pea roots and since no IAA appeared on alkaline hydrolysis of the ether extracts, the bicarbonate extracts or the tissue residues (except that accounted for by the known Salkowski reactive derivatives) it would seem that the most of the IAA which disappeared from solution was degraded, perhaps through the IAA oxidase system. It should be noted that if "bound auxin" is any compound in which IAA is combined with another molecule then indoleacetylaspartic acid much be regarded as bound auxin. It does not, however, correspond to the "auxin precursor" of Avery (3) in corn endosperm in which the IAA was released by very mild hydrolysis at pH 9 to 10 for 15 minutes at 100° C, conditions which have no hydrolytic action on indoleacetylaspartic acid.

The significance of these results in relation to growth cannot be evaluated at this stage as both elongating and non-elongating portions of the epicotyls were used in the studies. The correlation shown in figure 5 between the concentration of applied IAA which brings about maximum slit pea curvature, the concentrations which result in the maximal rate of indoleacetylaspartic acid formation and the concentration which initiates the appearance of free IAA may therefore be fortuitous especially as the tissues were incubated in water solutions of IAA for the curvature studies and in M/60 NaH_2PO_4 for studies on IAA uptake. However, if a similar relationship can also be demonstrated in elongating pea epicotyl sections, this correlation would lend support to the earlier suggestions (2) that the formation of indoleacetylaspartic acid (which has little biological activity when applied to pea epicotyls although this may possibly be due to failure to enter the tissues) is a detoxication process, the overloading of which produces deleterious effects.

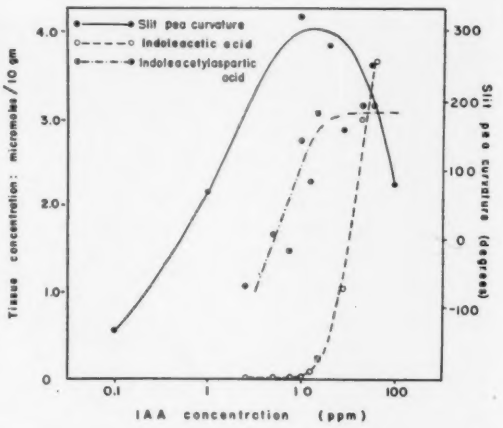

FIG. 5. The effect of the concentration of applied IAA on slit pea curvature and on the accumulation of IAA and of indoleacetylaspartic acid in pea epicotyls. The slit pea curvature test (4 replications) was carried out as described by Thimann and Schneider (10). The values for IAA and indoleacetylaspartic acid are those of figure 2.

SUMMARY

When pea epicotyls were incubated in IAA solutions containing M/60 NaH_2PO_4 about 20 % of the IAA disappearing from solution could be accounted for as Salkowski reactive substances in the tissues. These substances were mainly IAA and indoleacetylaspartic acid and their relative amounts depended on the concentration of IAA applied. If, however, tissues pretreated with IAA were placed in a moist chamber, practically all the IAA was converted into indoleacetylaspartic acid. Both the uptake of IAA and the formation of indoleacetylaspartic acid are aerobic processes. The observations on IAA uptake and metabolism are discussed in relation to their implications on IAA induced growth.

The authors wish to thank Dr. Norman E. Good for helpful discussions of the work and for synthesis of the indoleacetylaspartic acid.

Literature Cited

1. Albaum, H. G., Kaiser, S. and Nestler, H. The relation of hydrogen-ion concentration to the penetration of 3-indoleacetic acid into Nitella cells. Amer. Jour. Bot. 28: 513–518. 1937.
2. Andreae, W. A. and Good, N. E. The formation of indoleacetylaspartic acid in pea seedlings. Plant Physiol. 30: 380–382. 1955.
3. Avery, G. S., Bergen, J. and Shalucha, B. The total extraction of free auxin and auxin precursor from plant tissue. Amer. Jour. Bot. 28: 596–607. 1941.
4. Chantrenne, H. The requirement for coenzyme A in the enzymatic synthesis of hippuric acid. Jour. Biol. Chem. 189: 227–233. 1951.
5. Good, N. E., Andreae, W. A. and Van Ysselstein, M. W. H. Studies on indoleacetic acid metabolism. II. Some products of the metabolism of exogenous indoleacetic acid in plant tissues. Plant Physiol. 31: 231–235. 1956.
6. Reinhold, L. The uptake of indole-3-acetic acid by pea epicotyl segments and carrot disks. New Phytologist 53: 217–239. 1954.
7. Siegel, S. M. and Galston, A. W. Experimental coupling of indoleacetic acid to pea root protein in vivo and in vitro. Proc. Nat. Acad. Sci., U. S. 39: 1111–1118. 1953.
8. Sutter, E. Die chemische Bestimmung des Heteroauxins und Versuche über seine Aufnahme durch die Pflanze. Ber. schweiz. bot. Ges. 54: 197–244. 1944.
9. Tang, L. W. and Bonner, J. The enzymatic inactivation of indoleacetic acid. I. Some characteristics of the enzyme contained in pea seedlings. Arch. Biochem. 13: 11–25. 1947.
10. Thimann, K. V. and Schneider, C. L. Differential growth in plant tissues. Amer. Jour. Bot. 25: 627–641. 1938.

WAX DEPOSITS ON LEAF SURFACES[1,2]

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In an earlier paper, Mueller, Carr and Loomis (8) published details of an adaptation of the double-replica technique (12) for studying plant surfaces, and particularly for studying wax deposits on leaves. Surface waxes on plants may be of economic interest as industrial products, and they interfere with the adherence and penetration of various spray materials.

A number of theories of the nature of wax deposits on plants and of the method of their formation have been advanced since DeBary's early work (2). Kreger (5) reviewed the literature on wax to 1948, and published extensive studies of wax structure, as determined by x-ray diffraction. He considered the wax to be excreted in a liquid form, which hardens and partially crystallizes on the surface. Several investigators (1, 3, 9, 13, 14) have reported what they considered to be very fine canals through the cuticle of wax-forming leaves. Others (4, 7) have failed to find evidence of such canals.

Mueller et al (8) followed the development of wax on the leaf of *Musa* sp. and found no evidence of liquid wax. They observed pits in the cuticle that had a distribution similar to that of the wax columns, which may have been the openings previously considered to be canals. There was, however, no evidence that these pits extended through the cuticle. They concluded that wax is extruded as a softened paste under some pressure, and that this extrusion may be through the apparently intact wall and cuticle.

Linskens (6) studied dynamic contact angles of water and leaf surfaces as a quantitative measure of surface wetting; with an angle of zero for a completely wettable surface and an agle of 180° for a non-wettable surface. He obtained contact angles for several plant species. It may be noted from his results that all but one of the species known to be wax-bearing had contact angles greater than 120°, contrasted to angles of less than 80° for plants that do not normally show surface wax. Mueller et al (8) demonstrated that the concentration of wetting agent required to give complete surface wetting decreases with weathering of the surface wax. It is this effect of surface wax upon wetting that becomes economically important in the use of herbicidal, insecticidal, fungicidal, and nutritional sprays on plants.

Materials and Methods

Seedlings of corn (*Zea Mays*, single cross var. WF9 × M14) were used for a study of the effect of environment and age of leaves on wax deposits. Plants were started and grown in 4 environments: (a) 20° C in darkness, (b) 30° C in darkness, (c) an average of 25° C in daylight, and (d) of 20° C with

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[2] Journal paper No. J-2909 of the Iowa Agricultural Experiment Station, Ames, Iowa; projects 944 and 1139.

Fig. 1. Surface of embryonic leaf excised from the coleoptile; thin fibro-granular layer of pectins with a few fibrils of cellulose showing through.

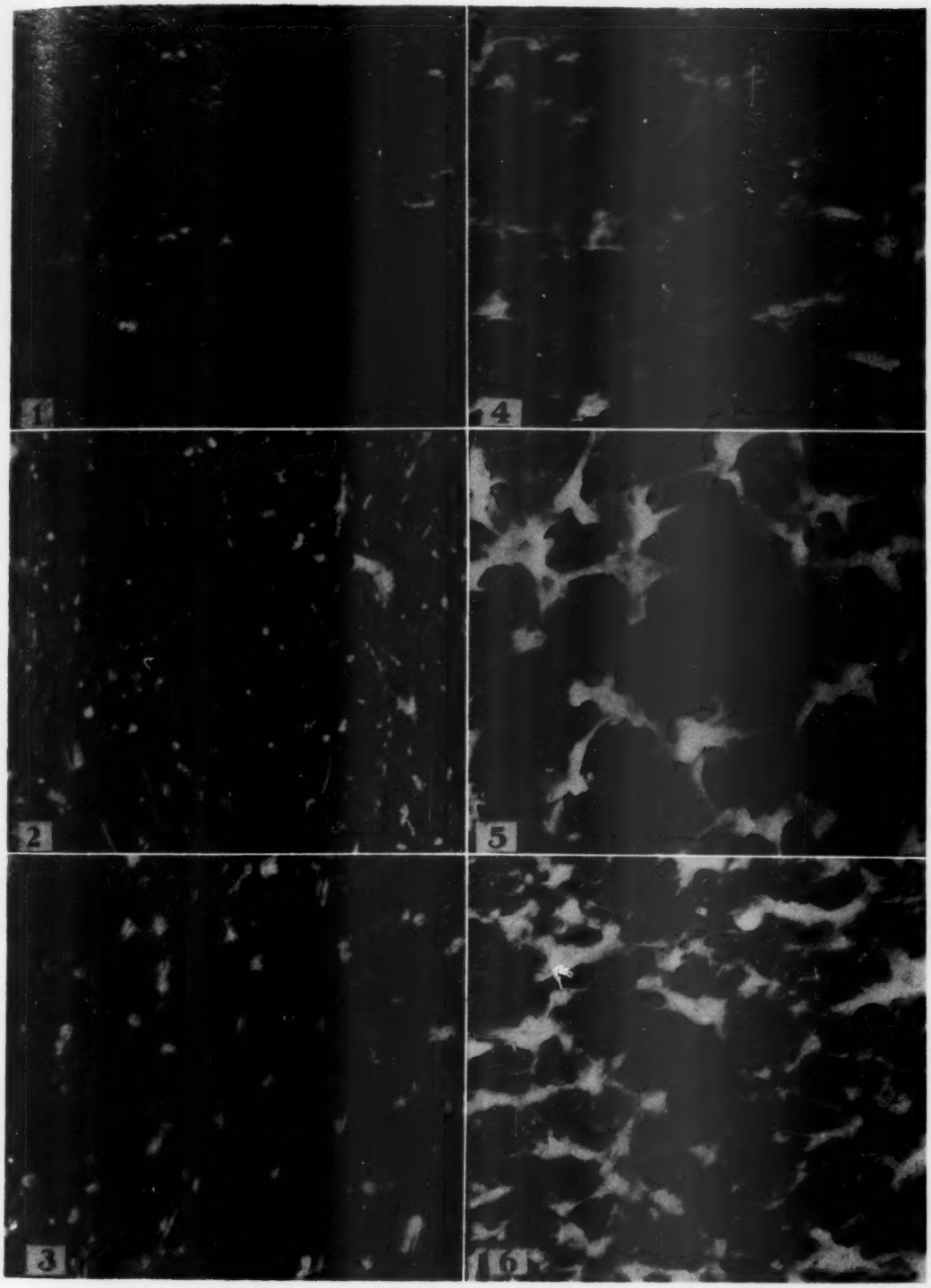
Fig. 2. Cuticle formation well advanced and beginning of wax extrusion in leaf just emerging from the coleoptile; plant grown in darkness at 30° C.

Fig. 3. Slightly later stage than figure 2; plant in greenhouse at about 25° C.

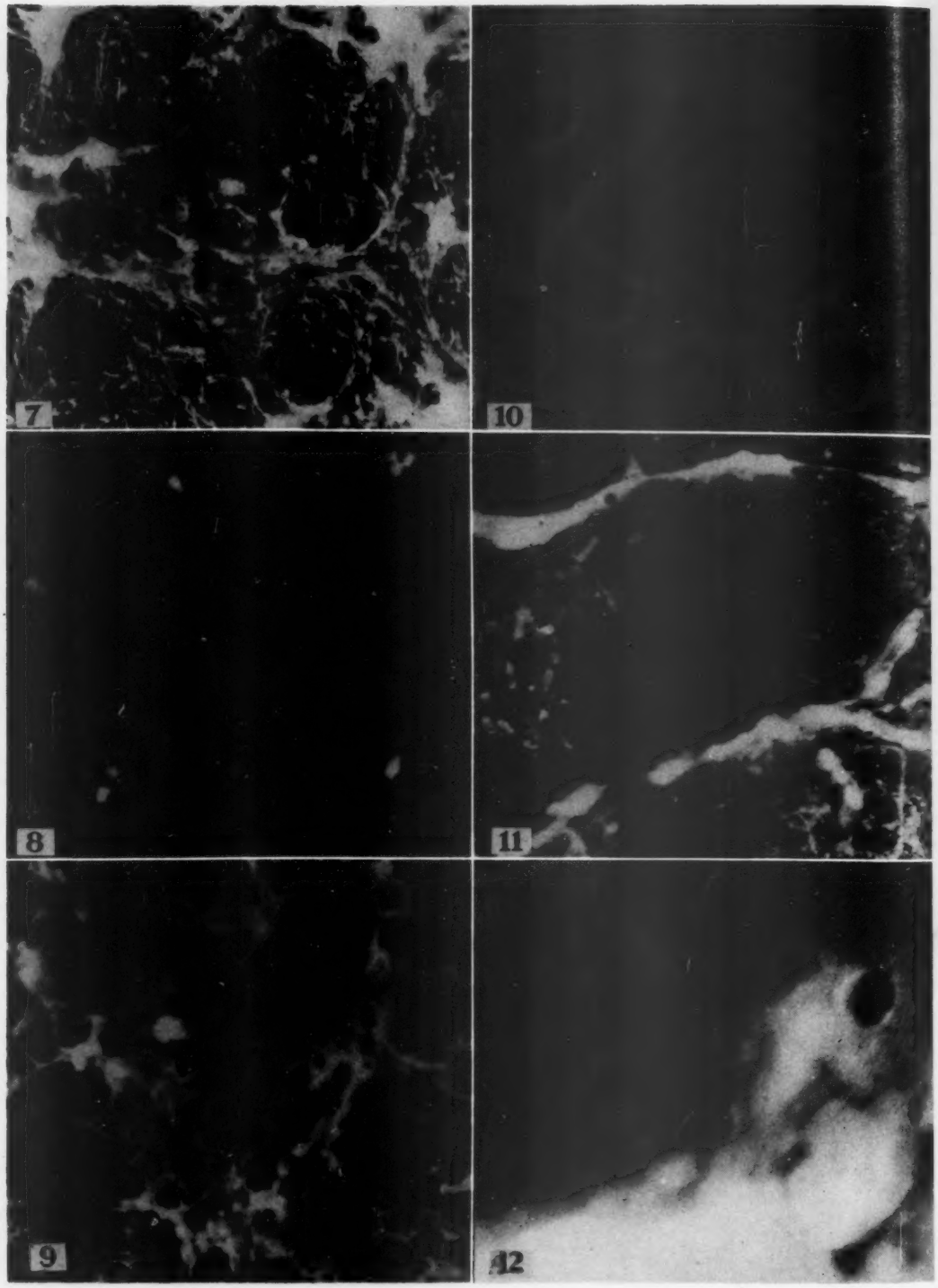
Fig. 4. Soft wax on expanding leaf grown at 20° C in darkness.

Fig. 5. High, rather sparse wax bodies on fully grown primary leaf at 30° C in darkness.

Fig. 6. Same as figure 5, but 20° C in darkness; wax appears softer and is scattered in smaller bodies.



FIGS. 1 to 6. Wax formation on primary leaves of *Zea Mays*. All magnifications approximately 14,000 diam.



FIGS. 7 to 9. Regeneration of wax on growing leaf of *Brassica oleracea*. Figure 7, 12,000×; figures 8 and 9, 5,600×.

high humidity and low light. Replicas were made from the primary leaves at 4 stages: 1) when the leaf was still in the coleoptile, 2) as it broke through the coleoptile, 3) leaf unrolled but still expanding, and 4) leaf just mature.

The regeneration of wax was studied on the leaves of cabbage (*Brassica oleracea* var. *capitata*). Uniform young leaves 1.5 inches (2.25 cm) in diameter were wiped with dry cotton to remove most of the wax. Leaves were harvested and replicas made at one or two day intervals following treatment. Comparable studies were made on senile leaves.

Isolated cuticle samples were prepared by incubating epidermal peels from *Agave* sp. in an aqueous solution containing 3 enzymes; 0.2 % partially purified pectinase, plus 0.2 % partially purified hemi-cellulase, and 0.5 % crude cellulase. The reaction mixture was incubated at 30° C with constant agitation for 5 days. The cuticle was then washed and replicas of the two surfaces were made in the usual manner.

An RCA, type EMU, electron microscope with a 50-kv potential was used for this study. Specimens were shadow-cast with gold at an angle of about 14°. Exposures were made at magnifications of 1,500× to 18,000× on medium contrast, 2×10-inch Kodak lantern slide plates. Unit sized intermediate negatives were made on Kodak Super Panchro-Press, Type B sheet film, and final prints were enlarged to give magnifications approximately twice the original. The plates are reduced slightly from the prints.

Experimental Results

No wax was present on the corn leaves excised from the intact coleoptile (fig 1), and the cuticle was in an early stage of development, as shown by its texture and by the cellulose fibrils that may be seen in the surface. No pits of the type found on *Musa* sp. and cabbage (8) were observed on corn at any stage of development. Wax appeared as the leaves broke through the coleoptile (fig 2), and development of the cuticle was proceeding rapidly. The wax was not crystalline at this stage, but it could have been either a thick liquid or a paste. Wax formation showed more variation with environmental conditions in stages 3, expanding, and 4, mature primary leaves. Wax appeared more solid in greenhouse plants (fig 3), and as though a soft material had been smeared in making the replicas when leaves were taken from the dark-grown plants (fig 4). The greatest difference shown at maturity was between 30° in darkness (fig 5) and 20° under the same conditions (fig 6). The wax formed at the higher temperature appeared firmer, and occurred in more scattered, much higher aggregates. Wax shown in figure 5 resembles that usually found on corn. Firmer wax at a higher temperature could be due to drying of a soft paste or to chemical changes in the wax molecules. Sugar supplies from the endosperm appeared to have been adequate in all of these seedling plants, since light and darkness did not cause significant changes in quantity or arrangement of wax.

In a study of the entire corn plant at silking, it was found that only the first 5 or 6 and possibly the uppermost 2 leaves developed surface wax while no wax could be observed on the central leaves. What may have been wax on the uppermost leaves was not as extensive as that on the lower leaves and had a much different appearance. These results did not appear to be an environmental effect.

Regeneration of wax was demonstrated on growing cabbage leaves. Figure 7 shows a check leaf as it appeared before dewaxing. Figure 8 shows a leaf that had just been dewaxed by rubbing with dry cotton. The granular appearance is not due entirely to shadow casting and suggests a smear of wax over the surface. Figure 9 shows the appearance of a similar leaf 14 days after dewaxing. No significant change was observed after this date. Total wax on the wiped leaves at the end of the experiment appeared to be less than that on the final checks by about the quantity removed in wiping. These results suggest that surface wax is excreted on this plant at a rather uniform rate for a period of perhaps two weeks, and that excretion is then stopped. This hypothesis was supported by tests with mature, older leaves. Such leaves showed no regeneration of wax.

Method of Wax Extrusion: Most writers on the subject of wax extrusion have either claimed to observe canals through the wall and cuticle, or have assumed their presence. We have been unable to find any trace of wax canals through the walls, either before or after wax deposition. Mueller et al (8) observed shallow pits on the leaves of Musa before wax extrusion started. Extrusion was in part near, but not in, the pits, and in part independent of them. Removal of wax from older leaves with wax solvents exposed unchanged pits but showed no breaks in the smooth cuticle where wax might have been extruded. We have repeated this work with cabbage. Our material showed shallow pits in groups (fig 10) when the wax was removed with cold xylene-chloroform mixture. There is no evidence that these are more than

Fig. 7. Wax on undisturbed young leaf.

Fig. 8. Wax removed by wiping with dry cotton.

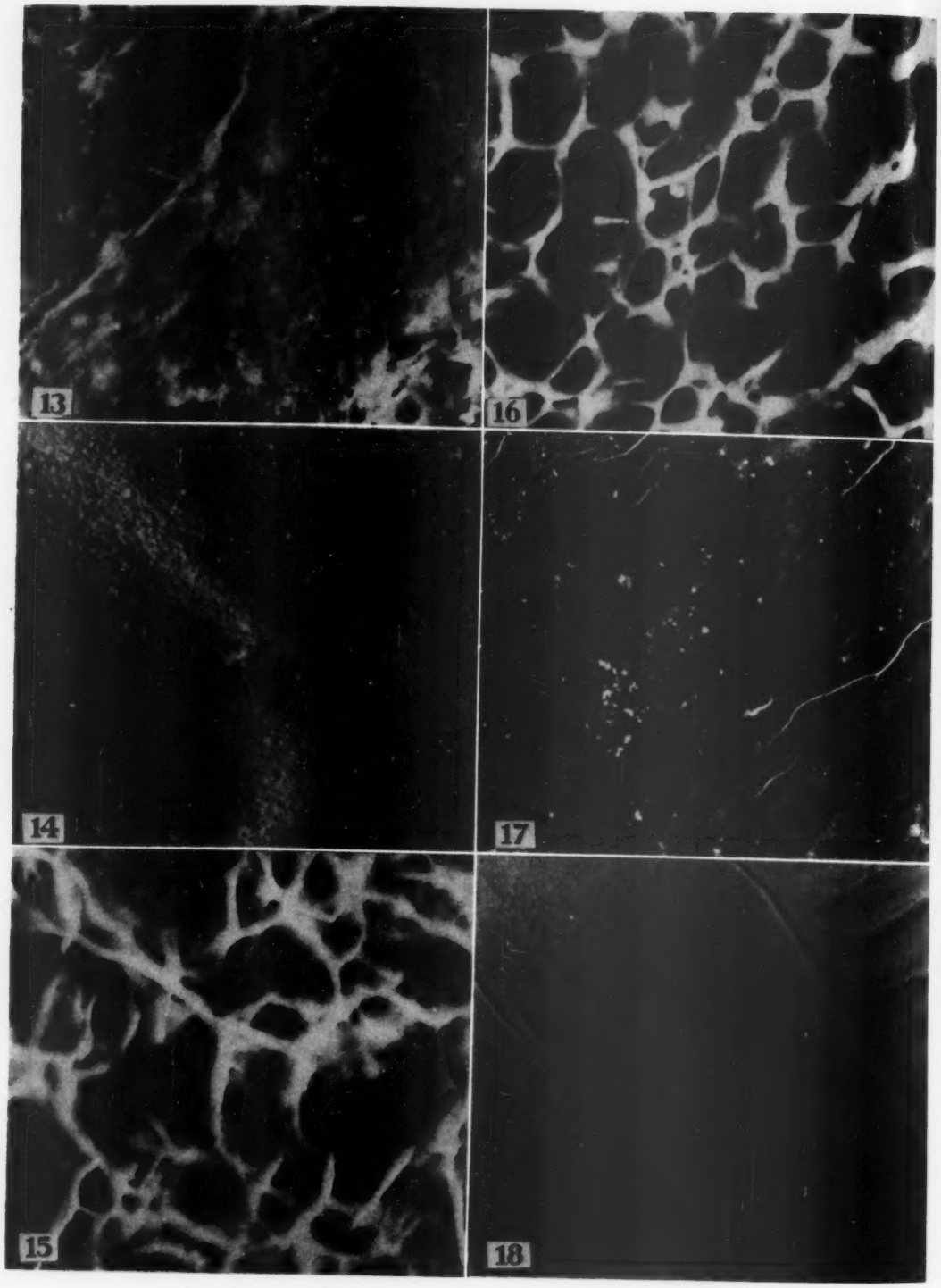
Fig. 9. Somewhat sparse wax deposits on wiped leaf 2 weeks after treatment.

Figs. 10 to 12. Pits on leaves. Figure 10, 26,000×; figure 11, 3,000×; figure 12, 36,000×.

Fig. 10. Pits on cabbage leaf after removing wax with solvent; both groups and small scattered pits were observed.

Fig. 11. Lower side of surface layer removed from *Agave* sp. by a pectinase-cellulase treatment; ridges are vertical walls of epidermal cells.

Fig. 12. Small pits in the inner surface of the epidermal wall shown in figure 11.



FIGS. 13 to 18. Miscellaneous. All 14,000× except figure 18, 5,600×.

surface pits with a well developed cuticle in the bottom.

In repeated tests we have never observed such pits on leaves of corn, so their presence is not required for surface wax formation. Corn leaves showing heavy wax deposits were refluxed for 2 hours in xylene-chloroform. Replicas of these leaves showed only a slight roughening of the smooth cuticle, and no evidence of canals or pits. These results show chemical differences between wax and cuticle, and indicate that a cuticle is formed under the wax deposits, presumably after extrusion.

Orgell (11) has published a method for isolating cuticle from leaves by enzyme action. When a mixed pectinase-cellulase enzyme was used on the epidermis of *Agave* sp. a fairly heavy, tough layer was separated out. A replica of the inner surface of the isolated layer is shown in figure 11. All of our tests indicated that the layer shown in the figure was the intact upper epidermal wall rather than the separated cuticle. Since this material had survived after 5 days of pectinase-cellulase treatment, it may be assumed that the entire wall had been impregnated with waxy or cutinaceous materials. It was interesting to note pits on the lower surface of this wall (fig 12) which were duplicates of those observed upon the outer surface of intact, dewaxed leaves. The cuticle of young cabbage leaves was removed in small, thin flakes by boiling in ethanolamine, a pectin solvent. This separation was not possible in older leaves, suggesting wax deposition between the cuticle and the underlying cellulase.

Wax Deposition and Epidermal Growth: Mueller et al (8) observed that the distribution of wax deposits on cabbage leaves was approximately constant while the leaves increased some 1200× in area, suggesting that new wax deposits are interposed between the older as the epidermal cells expand. Observations on corn leaves indicate another possible explanation of this distribution. Replicas of young, expanding primary leaves of corn showed definite bands of noncrystalline wax at the outer margins of the epidermal cells (fig 13). These bands were not observed on mature leaves, where the typical crystalline structure (cf fig 5) was continuous over the leaf. Figure 13 suggests that the upper walls of the epidermal cells grow from the margins only, and that wax deposits are laid during this period of local wall growth. Thus there would be no further change in the central wax deposits, because the cell does not grow in this area. Evidence of undifferentiated cuticle at the cell boundaries of a nonwaxy leaf of *Convolvulus arvensis* is shown in figure 14. The work on regeneration of wax in cabbage indicates that wax deposition continues for a longer period in this plant than is indicated for corn, but the same sequence of the initial establishment of a wax pattern which is not dispersed by growth is still possible. Since cell elongation in cotton has been shown to occur in a group of somewhat immature cellulose micro-fibrils (10), such a localization of enlargement might be expected to occur.

Wax Patterns: Closely related plants usually have similar wax patterns, but the patterns are sometimes very different, even between species of the same genus. Most grasses had a wax pattern similar to that of the lower leaves of corn (fig 5). This was not true of *Setaria lutescens* (fig 15), but was of *S. faberii* and *S. viridis*. *Panicum capillare* alone of the grasses studied showed no surface wax. The legumes as a group had a wax pattern typified by that of *Glycine Max* (fig 16). *Polygonum aviculare* had a wax pattern almost identical with that of the legumes. This was the only one of the several members of the Polygonaceae studied that showed surface wax. The surface patterns found on different species varied widely. It is suggested that the wax pattern depends upon the structure of the cuticle, the pathway of wax through the cuticle, and the chemical constituents of the wax. Two leaves on which wax has not been observed are shown in figures 17 and 18. Folding of the cuticle is common in mature leaves of many species, and suggests that the primary cuticle is a very thin layer. Folding was observed on both waxy and nonwaxy leaves. A list of the leaves that have been studied is given below with a classification on the basis of the presence or absence of surface wax.

Species on which surface wax has or has not been observed:

Wax-bearing	Non wax-bearing
Zea Mays	*Panicum capillare*
Setaria faberii	*Solanum rostratum*
Setaria viridis	*Solanum nigrum*
Setaria lutescens	*Solanum tuberosum*
Digitaria sanguinalis	*Lycopersicum esculentum*
Saccharum officinarum	*Petunia violacea*
Amaryllis vittata	*Nicotiana Tabacum*
Brassica oleracea	*Gossypium hirsutum*
Nicotiana glauca	*Ambrosia trifida*
Glycine Max	*Ambrosia elatior*
Trifolium repens	*Polygonum persicaria*
Trifolium pratense	*Xanthium* sp.
Melilotus alba	*Ipomoea purpurea*
Pisum sativum	*Convolvulus arvensis*
Lathyrus odoratus	*Cirsium arvense*
Polygonum aviculare	*Cirsium vulgare*
Berberis thunbergii	*Plantago lanceolata*
Lonicera sp.	*Plantago major.*
Musa sp.	*Asplenium nidus*

Fig. 13. Zones over and on either side of vertical epidermal cell wall; considered to be possible regions of cell enlargement in these growing cells.

Fig. 14. "Growing" zone on small leaf of *Convolvulus arvensis*.

Fig. 15. Wax pattern on leaf of *Setaria lutescens*.

Fig. 16. Wax pattern on *Glycine Max;* typical of legumes.

Fig. 17. Folded cuticle and absence of wax on mature leaf of *Cirsium vulgare*.

Fig. 18. Stomate and folded cuticle on wax-free leaf of cotton, *Gossypium hirsutum*.

Wax-bearing	Non wax-bearing
Rosa carolina	*Mesembryanthemum cordifolium*
Kalanchoë pinnata	
Kalanchoë marmorata	
Tropaeolum majus	
Aloe sp.	
Agave sp.	
Picea pungens	
Mesembryanthemum lingua	
Peperomia sp.	

Discussion

Surface wax deposits of various semi-crystalline or amorphous structure have been observed on about half of the leaves studied. There is no clear-cut correlation between surface wax and xeromorphic adaptation, so that the presence or absence of wax appears to be of doubtful survival value. This is to say that a continuous cuticular layer, rather than scattered surface wax deposits, is the major factor in water conservation by epidermal cells.

Surface wax appears to be deposited only on young leaves, and essentially only during or shortly after the period of leaf expansion. Deposition is probably related to the development and solidification of the cuticular layer. Since we have been unable to find any evidence of wax channels, or any residual effect of the passage of the wax through the wall and cuticle, extrusion must be in the form of a soft paste or a semi-liquid; such extrusion ending when the wall, the cuticle, or both, become so solidified as to stop movement of this type. We consider it significant in this connection that a smooth, apparently uniform cuticle is formed under the heaviest surface wax deposits. The probability that wax formation may continue in older leaves, with the later formed material intruded into the cellulose wall below the cuticle, is being investigated. Evidence for such intrusion is given by the fact that the cuticle of a young cabbage leaf could be removed in flakes by cooking with ethanolamine, while that of an older leaf could not.

We visualize the formation of what we term the primary cuticle as the flooding of a thin layer of pectin on the outer surface of the epidermis with a substance analogous to a drying oil. The solidification of this material forms the primary cuticle as a very thin layer. Such a layer may still be effective in conserving water if it is continuous and sufficiently impermeable to water. Since the cuticle of young leaves is normally more pervious to water than that of older leaves, one or both of these conditions may develop slowly. We consider that the final hardening of the primary cuticle represents the termination of surface wax extrusion. Further wax deposits could then form below the cuticle or be intruded into the cellulose at any level. Presumably the development of the very thick cuticular layers characteristic of some leaves is due to the deposition of cutin, wax or a mixture of these substances on the under surface of the primary cuticle. These secondary layers may actually resemble annual growth layers in some perennial leaves.

Although a smooth cutin layer in mature cuticle may be effective in reducing cuticular transpiration, it does not compare with surface wax in resisting wetting by sprays with a high surface tension. Wax deposits will thus have an economic interest in the application of herbicides and other sprays, and can be a major factor in the differences in spray retention that are sometimes utilized in selective herbicidal action, with mustard in peas, for example.

The suggestion that the upper walls of epidermal cells grow from the margins merits further attention. Considerations of both the mechanism of cell enlargement (10) and of the greater susceptibility of young leaves to herbicidal sprays are involved. If an immature zone around the margins of the expanding epidermal cells is responsible for a major portion of the greater susceptibility of young leaves to herbicides, there should be a sharp reduction in susceptibility with the end of growth in any given leaf.

Summary

Electronmicrographs are shown which depict the development of the cuticle and the deposition of surface wax on the primary leaves of maize seedlings. There is no evidence of the formation of permanent or semi-permanent wax canals during deposition. The removal of wax deposits from older leaves exposes a smooth, uniform cuticle beneath the wax. Wax extrusion continued for some days in growing cabbage leaves but was not demonstrable in mature, older leaves.

Wax patterns on growing leaves suggest that the upper wall of epidermal cells enlarges at the cell margins. Possibly the greater susceptibility of growing leaves to herbicides is related to a permeable, immature zone in the cuticle through which the herbicide can penetrate rapidly.

A total of 48 plant species has been examined. Although some of these were selected because they appeared to be wax-bearing, only 28 of the species showed surface wax deposits. Such wax deposits do not appear to play an important role in the water economy and survival of the plant. They do, however, reduce the retention of sprays on leaves, and thus affect the action of herbicidal and other sprays.

We are indebted to Messrs. G. L. Cowgill and M. D. Blue for technical assistance in the operation of the electron microscope.

Literature Cited

1. Burger, D. Beiträge zur Lebensgeschichte der *Populus tremula* L. Pp. 1–79. H. Rütschii, Zürich 1920.
2. DeBary, A. Ueber die Wachsüberzüge der Epidermis. Bot. Zeit. 29: 128–175, 566–619. 1871.
3. Dous, E. Ueber die Wachsausscheidungen bei Pflanzen. Bot. Arch. 19: 481–473. 1927.
4. Fritz, H. Ueber die Cuticula von Aloë und Gasteria Arten. Jahrb. wiss. Bot. 81: 718–746. 1935.
5. Kreger, D. R. An x-ray study of waxy coatings from plants. Rec. trav. bot. néerl. 41: 603–736. 1948.

6. Linskens, H. F. Quantitativ Bestimmung der Benetzbarkeit von Blattoberflächen. Planta 38: 591–600. 1951.
7. Meyer, Madeleine Die submikroskopische Struktur der kutinisierten Zellmembranen. Protoplasma 29: 552–586. 1938.
8. Mueller, L. E., Carr, P. H. and Loomis, W. E. The submicroscopic structure of plant surfaces. Amer. Jour. Bot. 41: 593–600. 1954.
9. Nieuwenhuis van Uexküll-Guldenband, M. Sekretions-Kanäle in der Cuticularschichten der extrafloralen Nektariën. Rec. trav. bot. néerl. 11: 291–311. 1914.
10. O'Kelley, J. C. and Carr, P. H. Elongation of the cotton fiber. In: Growth and Differentiation in Plants, W. E. Loomis, ed. Pp. 55–68. Iowa State College Press, Ames, Iowa 1953.
11. Orgell, W. H. The isolation of plant cuticle with pectic enzymes. Plant Physiol. 30: 78–80. 1955.
12. Schwartz, C. M., Austin, A. E. and Weber, P. M. A positive replica technique for electron microscopy. Jour. Appl. Physics 20: 202–205. 1949.
13. Wijnberg, A. Over rietwas en de mogelijkheden zijner technische gewinning. Pp. 1–198. Debussy, Amsterdam 1909.
14. Ziegenspeck, M. Ueber das Ergusswachstum des Kutins bei Aloë Arten. Bot. Arch. 21: 1–8. 1928.

BRIEF PAPERS

THE PHYSICAL NATURE OF TRANSPIRATIONAL PULL[1]

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Though there seems to be rather general agreement (with few exceptions[2]) among plant psysiologists that the Cohesion Theory of the ascent of sap best agrees with the known facts, the nature of the initiating force is not so generally agreed upon. Consequently, to be on the safe side, it is usually referred to as the "transpirational pull," without attempting to explain the physical basis of this "pull." In most modern texts, an attempt has been made to relate it to diffusion. Thus, according to Meyer and Anderson (6):

> Within the lamina of the leaf, gradients of diffusion pressure deficits, gradually increasing in magnitude from cell to cell in the direction in which water is moving are established between the xylem ducts and the cells from which evaporation is occurring. Water therefore moves from a given vessel or tracheid into adjacent cells, which results in the development of a tension in the water column occupying that element of the xylem.

The same concept, in different words, is to be found in Curtis and Clark (4), Bonner and Galston (2), and even Maximov (5). This implies that the transpirational pull really involves a push in the top fraction of a millimeter of the column (from the top of the xylem through the living cells, to the evaporation surface of the mesophyll cells) which initiates a pull below it. Thus the cohesive force would be responsible for the rise of the whole column except this uppermost fraction of a millimeter.

This would also mean that the cohesive pull is initiated not at the water surface, but somewhere back of this—in other words, instead of raising itself by hanging on to the inner surface of the cell wall, the column would have to pull itself up by its own bootstraps! It is difficult to understand the popularity of this theory. It certainly does not follow the principle of Occam's razor since it replaces a simple concept by a complex one. Furthermore, the Askenasy experiment cannot be explained in this way since no living cells are involved. In this case, at least, the pull must be initiated right at the surface, rather than a fraction of a millimeter below, a fact that is recognized by Meyer and Anderson.

The usual point of view seems to be that if the diffusion gradient is in the right direction, then diffusion must account for whatever flow that occurs. Actually, what must be demonstrated is that the *rate of diffusion of the water in the liquid state from the vessels to the mesophyll cells is adequate to keep up with the rate of diffusion of the water vapor from the mesophyll cells into the air.* This point can be cleared up by a consideration of Fick's law of diffusion:

$$\frac{s}{t} = Da\,\frac{C_1 - C_2}{x}$$

where s = amount of substance diffusing
t = time
D = coefficient of diffusion
a = area across which diffusion is occurring
C_1 = higher concentration
C_2 = lower concentration
x = distance separating C_1 from C_2

The question now is, whether or not the ratio s/t for the diffusing water vapor is of the same order as s/t for the diffusing liquid water—i.e., it is required to prove that

$$\frac{s/t\ \text{(water vapor)}}{s/t\ \text{(liquid water)}}$$

is of the order of 1 in a transpiring leaf, in which rate of water loss from the leaf is just compensated by rate of water intake.

Let us assume a commonly found set of conditions: a turgid plant with open stomata in an atmosphere of 60 % relative humidity, the mesophyll cells having an osmotic potential of 15 atms and maintaining their turgor unchanged (i.e., rate of water absorption by mesophyll cells = rate of water loss). The problem can be broken down into four parts.

a) What is the ratio

$$\frac{D\ \text{(water vapor)}}{D\ \text{(liquid water)}}\ ?$$

The coefficient of diffusion of water vapor is 0.22 cm^2/sec, the value for liquid water is of the order of 2.2×10^{-5} cm^2/sec, assuming that it is about the same as that for the physically similar HCl in water (see International Critical Tables). Therefore

$$\frac{D\ \text{(water vapor)}}{D\ \text{(liquid } H_2O\text{)}} = 10^4$$

b) What is the ratio

$$\frac{C_1 - C_2\ \text{(water vapor)}}{C_1 - C_2\ \text{(liquid water)}}\ ?$$

The actual concentrations of the liquid and gaseous water cannot be determined, but the quantity that is more important in diffusion—the activities—can be indirectly measured by means of the osmotic quantities which are related to the activities of the water molecules.

Since the mesophyll cells have an osmotic potential of 15 atms, their relative humidity is about 99 %, or 39 % above that of the atmosphere. $C_1 - C_2$ (water

[1] Received November 2, 1953.

[2] See Scholander et al (9).

vapor) is therefore about 600 atms (since 1 % r.h. is equivalent to about 15 atms). Since the mesophyll cells are turgid, $C_1 - C_2$ (liquid water) is less than the difference between the osmotic potential of the mesophyll cells and that of the vessel sap. It therefore cannot be more than about 10 atms. Therefore

$$\frac{C_1 - C_2 \text{ (water vapor)}}{C_1 - C_2 \text{ (liquid water)}} = \frac{600}{10} = 60$$

c) What is the ratio

$$\frac{x \text{ (water vapor)}}{x \text{ (liquid water)}} ?$$

Since C_1 (water vapor) is taken as the concentration at the surface of the mesophyll cells and C_2 as that in the external atmosphere, x (water vapor) will be the shortest distance between the mesophyll cells and the external atmosphere. This must be the distance from the surface of the mesophyll cells adjacent to the substomatal cavity to the outer surface of the leaf (or a little above it). Again, since C_1 (liquid water) is that of the vessel sap, and C_2 (liquid water) that of these same mesophyll cells adjacent to the substomatal cavity, x (liquid water) will be the distance across the cells between these two—a distance at least as great as the above which is equivalent to about two cells. Therefore

$$\frac{x \text{ (water vapor)}}{x \text{ (liquid water)}} = 1 \text{ (app.)}$$

d) What is

$$\frac{a \text{ (water vapor)}}{a \text{ (liquid water)}} ?$$

Since a is the area across which diffusion is occurring, a (water vapor) for a single leaf must be equal to the total stomatal area of that leaf and a (liquid water) the total area of the vessels in the leaf at right angles to the direction of flow to the stomata. Since, however, we are dealing with small apertures in both cases, the diffusion rate is proportional to the perimeters rather than the areas. This means that the effective area for diffusion of the water vapor is the whole surface of the leaf. Since the effective area for diffusion of the liquid water cannot be any greater than this, the ratio

$$\frac{a \text{ (water vapor)}}{a \text{ (liquid water)}} = 1 \text{ (at the least)}$$

Therefore, from a), b), c), and d),

$$\frac{s/t \text{ (water vapor)}}{s/t \text{ (liquid water)}} = 10^4 \times 60 \times 1 \times 1 = 6 \times 10^5 .$$

We are forced to conclude, then, that the rate of diffusion of water vapor from the leaf into an atmosphere of 60 % relative humidity is at the very least 600,000 × the rate of diffusion of the liquid water from the vessels to the mesophyll cells in a normal, turgid, leaf with open stomata and with an osmotic potential of 15 atms in the mesophyll cells. And this does not take into account the retarding effect of the plasma membrane on the diffusion of water through the living cells.

The diffusion of water in the liquid state is obviously far too slow to play any role whatever in the ascent of sap from the vascular stream to the mesophyll cell surface. Perhaps the main reason for suggesting this concept is the idea that surface forces are inadequate. Thus Preston (8) states:

> No mention need therefore be made here either of the conception of atmospheric pressure as the driving pressure (since trees are often higher than the barometer column in water) or of surface tension.

Even physics texts sometimes give the same impression. In his discussion of capillarity Stewart (11) states:

> Strictly speaking, the water is not lifted by the surface film. The upward force of the film causes a decrease in the pressure under the surface; above the surface the pressure is that of the atmosphere. Hence in the liquid under the film the pressure will be less than the atmospheric pressure, and the liquid will be forced up by the pressure on the outside of the tube.

On this basis, the maximum height attainable due to capillarity is 30 ft (i.e., that due to atmospheric pressure). That surface forces are able to exceed this has long been known from the Askenasy experiment. In fact, if this concept were correct, no capillary rise could occur in a vacuum (a point that is very easily disproved).

Similarly, Adam (1) states that

> The liquid is not pulled up the tube by a hypothetical surface tension pulling on the walls, as is suggested by the explanation found in so many elementary textbooks—it has never been made clear what is the hook on the wall to which this "surface tension" attaches itself, nor how the hook contrives to move up the tube in advance of the rising meniscus. . . . the pressure difference follows from the free energy resident in the surface, and the liquid then flows up the tube under the hydrostatic pressure.

Older physics texts do seem to recognize that surface tension may cause a capillary rise above that due to atmospheric pressure alone. Thus, Millikan (7) states:

> Hence, unless the ratio of the cohesion to the adhesion exceeds a certain limit, a thin film of the liquid must spread indefinitely over the surface of the solid.

Perhaps the only modern physiology text that considers the transpirational pull to be a surface force is that by Thomas (12). Crafts et al (3) clearly connect the two.

As Millikan points out, the capillary force is dependent on and even initiated by the adhesion between the liquid and the walls of the capillary tube. In the case of the evaporating surface of the mesophyll cells, this adhesive force would be the imbibitional force of the cell wall. That this force is adequate to account for the ascent of sap is obvious from the fact that imbibition pressures of as high as about 1000 atms have been measured (Shull, 10). Furthermore, this

pressure rises rapidly with dehydration of the cell wall, and would therefore increase the tension as the transpiration exceeded the absorption rate.

Thus a consistent concept of the Cohesion Theory requires that the initiating (adhesive or imbibitional) force is located at the evaporating surface and that the cohesive force is transmitted from here all the way back through the living leaf cells to the vessel, down the vessels to the living root cells, all the way to the soil particle in contact with the absorbing root surface.

The process can be visualized on a molecular basis something as follows. The cell wall particles are all hydrated. If some water molecules evaporate from the particles at the outer surface of the mesophyll cell walls, these particles will attract water with a greater adsorptive force than the ones directly below them and will therefore adsorb some water molecules from them. Due to the cohesion of the water molecules, this will result in a rise of the column as a whole (if the force is large enough to overcome the gravitational and frictional forces).

Curtis and Clark (4) have objected to this concept from another point of view. By use of Poiseuille's Law, they have concluded that the force required to move water through the pores in the cell wall would require 100,000 atms for pores 0.1 μ in diameter. That their conclusion is in error can be demonstrated by use of a Seitz filter. With less than an atmosphere pressure, it is possible to obtain a flow through one of these filters at least 100 times as rapid as that due to transpiration from a leaf of equal area, though the pores are small enough to hold back organisms of about 0.1 μ in diameter.

Curtis and Clark's reasoning is based on the "assumption that a head of water of 1 m supplies water fast enough to supply the leaves when the vessels are 0.1 mm in diameter." In other words, they are assuming that the capillaries extend the whole length of the plant. But, if the limiting capillaries are simply those in the wall of a mesophyll cell, the length may be the thickness of the wall or about 1 μ—i.e., 10^{-7} times the length of the vessels in a plant 10 m high. Since the $P \propto L$, the minimum pressure needed to initiate a flow through 0.1 μ pores in a mesophyll cell wall would be only 0.01 atm instead of 100,000 atms.

The calculations for a leaf can be readily made. According to Poiseuille's Law:

$$P = \frac{8Vnl}{\pi r^4}$$

where P = difference in pressure at the two ends of the tube
V = volume flowing out of tube in unit time
n = 0.01 poise for water
l = length of the tube
r = radius of the tube

The main basis of Curtis and Clark's argument is that $P \propto (l/r^4)$. But this is counteracted by the extremely small value of V for a single pore (or, put in another way, the very large number of pores involved). Thus V = volume moving through 1 pore

$$= \frac{\text{volume moving through 1 leaf}}{\text{number of pores per leaf}}$$

Let us assume a) the extraordinary rate of unity for transpiration (1 ml/cm^3 × hr). In cgs units this becomes (1/3600 ml)/(cm^3 × sec). Let us also assume b) that the number of pores per leaf = total cell surface/2 × the area of a single pore (i.e., that half the cell surface is pores), and c) that the cells are spherical. Actually the cells are irregular and have much more specific surface than this, but the error will be approximately canceled by the fact that the cells are in contact for part of their surface and, therefore, the whole surface is not available for evaporation.

From the above assumptions, the total cell surface = leaf volume times specific cell surface

$$= \frac{3 \times \text{leaf vol}}{r}$$

The number of pores (assumption b), if the area of one pore is 1 μ^2 or 10^{-8} cm^2,

$$= \frac{3 \times \text{leaf vol}}{r} \times \frac{1}{2 \times 10^{-8}} = \frac{1.5}{10^{-8} r}$$

$\therefore$ V (moving through 1 pore)

$$= \frac{1/3600}{1.5/10^{-8} r} = \frac{10^{-8} r}{5400} \text{ ml/sec}$$

If we assume the length of the pore = 10 times the radius (e.g., 1 μ long and 0.1 μ radius),

$$P = \frac{8 \times (10^{-8} r/5400) \times 10^{-2} \times 10r}{\pi r^4}$$

$$= \frac{0.5 \times 10^{-12}}{r^2} \text{ dynes/cm}^2$$

If the pores are 0.1 μ in radius (half the radius assumed by Curtis and Clark,

$$P = \frac{0.5 \times 10^{-12}}{(10^{-5})^2}$$

$$= 0.5 \times 10^{-2} \text{ dynes/cm}^2$$

$$= 0.5 \times 10^{-8} \text{ atms.}$$

This value is, of course, only an approximation based on assumptions that greatly simplify the situation. However, the errors tend to cancel each other out and even if the assumptions led to an appreciable error, the value would still be insignificant. Even rough calculations readily reveal that the pressure required to produce the flow must be insignificant, for pure water moving under its own weight flows through the finest filter paper at a much more rapid rate per unit area than its most rapid loss from a leaf by transpiration—even when it covers the filter paper by a layer only 1 mm thick. Since the volume of water on each cm^2 is then only 0.1 ml, the pressure due to the weight of the water is 100 dynes/cm^2 = 10^{-4} atm. Yet the pores of the finest filter paper are small

enough to hold back the smallest bacteria, and the distance l is at least 100 times greater than that of the cell wall's pores.

Summary

1. Physiology texts state that the "transpirational pull" initiates the ascent of sap by means of a diffusion of liquid water from the top of the vessels in the leaf to the evaporating surface of the mesophyll cells. This concept is shown to be impossible because under commonly found conditions, the diffusion rate of the water vapor may be 600,000 times that of the liquid water.

2. The objection of Curtis and Clark that it would require a pressure of 100,000 atms to produce a flow through the microcapillaries of the cell wall is also shown to be in error. Actually, less than 0.5×10^{-8} atms would be needed.

3. A consistent and physically sound theory of the ascent of sap is possible only if it is assumed that surface tension forces initiate the rise—i.e., that the adhesive (imbibitional) forces are increased at the surface due to evaporation and this causes the rise of the whole column due to cohesion between the water molecules.

Literature Cited

1. Adam, N. K. The Physics and Chemistry of Surfaces, 2nd ed. Pp. 1–402. Clarendon Press, Oxford 1938.
2. Bonner, J. and Galston, A. W. Principles of Plant Physiology. Pp. 1–499. W. H. Freeman, San Francisco, California 1952.
3. Crafts, A. S., Currier, H. B., and Stocking, C. R. Water in the Physiology of Plants. Pp. 1–240. Chronica Botanica, Waltham, Massachusetts 1949.
4. Curtis, O. F. and Clark, D. G. An Introduction to Plant Physiology. Pp. 1–751. McGraw-Hill Book Co., New York 1950.
5. Maximov, N. A. A Textbook of Plant Physiology. Pp. 1–381. McGraw-Hill Book Co., New York 1930.
6. Meyer, B. S. and Anderson, D. B. Plant Physiology, 2nd ed. Pp. 1–784. D. Van Nostrand Co., New York 1952.
7. Millikan, R. A. Mechanics, Molecular Physics and Heat. Pp. 1–242. Scott, Foresman & Co., Chicago, Illinois 1902.
8. Preston, R. C. Movement of water in higher plants, Chapter 4. In: Deformation and Flow in Biological Systems, A. Frey-Wyssling, Ed. Pp 257–321. Interscience Publishers, New York 1952.
9. Scholander, P. F., Love, Warner E., and Kanwisher, John W. The rise of sap in tall grapevines. Plant Physiol. 30: 93–104. 1955.
10. Shull, C. A. Measurement of the surface forces in soils. Bot. Gaz. 62: 1–31. 1916.
11. Stewart, O. M. Physics. Pp. 1–750. Ginn & Co., Boston, Massachusetts 1939.
12. Thomas, M. Plant Physiology, 3rd ed. Pp. 1–504. J. & A. Churchill, London 1949.

EFFECT OF RIBONUCLEASE ON SALT ABSORPTION BY EXCISED MUNG BEAN ROOTS[1]

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The transport of ions into plant root cells is generally believed to be by means of ion-binding carrier compounds. Several types of compounds have been proposed as carriers, but none has won universal support (6). Recently, from observations on the effect of ribonuclease on absorption of calcium by Elodea cells and from cytological evidence, Lansing and Rosenthal (4) have suggested that ribonucleic acid may function as an ion-binding carrier compound during salt absorption. The results reported here lend support to their proposal.

In this study, mung bean (*Phaseolus aureus*) were germinated and grown at 25° C with roots in an aerated 10^{-4} M $CaCl_2$ solution which was changed daily. The first centimeter of root tip was excised from 3-day-old seedlings. Fifty root tips were placed in a beaker, washed, and treated with a solution of crystalline ribonuclease (100 μgm/ml) for short periods at 25° C. The enzyme was preheated at 70° C for 20 minutes before use. Control roots were similarly treated but without the enzyme. After enzymic treatment the roots were washed and placed in a 10^{-4} M solution of either RbCl with Rb^{86} or KH_2PO_4 with P^{32}. The activities of the solutions were less than 5 μc/l. In some cases the solutions contained $Ca(NO_3)_2$ of 10^{-3} M. The roots were allowed to absorb Rb or phosphate for 30 min at 25° C under vigorous aeration. After the absorption period, the roots were washed several times with inactive salt solution of 10^{-2} M and water and dried at 100° C. The radioactivity of the roots was determined in the conventional manner.

Typical results showing the effect of ribonuclease on Rb and phosphate absorption are presented in table I. The data are the means of duplicate determinations. The experiment has been repeated several times with similar results. The data indicate that there was a very marked effect of the enzyme on Rb absorption by mung bean roots. The enzymic effect was apparently influenced strongly by the presence of Ca in the absorption medium. In the absence of Ca in the absorption medium, the ribonuclease pretreatment enhanced the uptake of Rb by roots. The absorption was linear up to one hour. Rb uptake in the

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TABLE I

EFFECT OF RIBONUCLEASE ON RUBIDIUM AND PHOSPHATE ABSORPTION BY EXCISED MUNG BEAN ROOTS

PRETREATMENT TIME WITH ENZYME	CALCIUM IN ABSORPTION MEDIUM	ABSORPTION	
		RUBIDIUM	PHOSPHATE
min	*M*	*cps*	*cps*
No pretreatment	0	8.4	4.2
10	0	14.2	3.6
20	0	19.6	2.7
No pretreatment	10^{-3}	20.4	13.4
10	10^{-3}	8.0	9.3
20	10^{-3}	4.2	8.3

presence of Ca, however, was depressed by the pretreatment. Similar results were obtained with 10^{-3} M $MgSO_4$; but $NaNO_3$ of 10^{-3} M had little or no effect. These results indicate an interaction between the effects of ribonuclease and polyvalent cations. The absorption of phosphate, on the other hand, was less affected by the enzyme and was decreased both in the absence and presence of Ca by the pretreatment. The depressing effect of Ca on Rb absorption by enzyme-treated roots is interesting in view of previous reports (7, 8) that Ca enhances salt uptake by normal roots. This can also be seen in the data in table I which show a marked stimulation by Ca of both Rb and phosphate absorption by control roots.

From the results presented in table I, it appears that ribonucleic acid, or more probably a ribonucleoprotein, could be involved in ion transport during salt absorption by plant roots. Lansing and Rosenthal (4) have cited several chemical and cytological evidences supporting the role of ribonucleic acid as an ion-binding carrier compound. Tanada (7) has recently shown that Rb absorption by excised mung bean roots is markedly affected by ultraviolet radiation of 2537 Å, a wavelength which is strongly absorbed by nucleic acids. It is realized, however, that all these evidences are indirect in nature; but they could be utilized for obtaining direct evidence on the identity of ion transport compounds.

Additional support for the contention of Lansing and Rosenthal (4) that some ribonucleic acid is localized on cell surfaces was obtained by repeating their technique of staining with toluidine blue after ribonuclease treatment. According to Heller and Kopac (2), toluidine blue is specific for ribonucleic acid. In this investigation, excised mung bean roots were pretreated for 20 min with a ribonuclease solution (100 μgm/ml). The roots were washed and placed with control roots in a toluidine blue solution of 10 ppm for 5 min. The stained roots were then washed with water. It was easily apparent that the enzyme-treated roots stained less than control roots. Microscopic observation showed some staining localized near the surfaces of cells. Incidentally, Lundegårdh has commented on the presence of nucleotides in cell surfaces (5). It is possible that the action of ribonuclease might not be localized only at the surface of cells. Recent investigations by Kaufmann and Das (3) and by Brachet (1), using longer time of treatment or greater concentration of enzyme, suggest that ribonuclease can enter into cells. These results, however, do not exclude some ribonuclease action near the surfaces as it enters into the cell.

If the effects of ribonuclease reported in this paper were due to degradation of ribonucleic acids near the external surfaces of root cells, there is a possibility that some of the degraded products of the nucleic acids might diffuse outward into the surrounding medium. With the object of exploring this possibility, after the usual enzymic treatment of roots the ribonuclease solution was analyzed spectrophotometrically in the ultraviolet with a Beckman DU spectrophotometer. The solution showed two absorption maxima with the largest in the 250 to 260 mμ region and the other in the 290 to 300 mμ region. The absorption in the wavelength region of 250 to 260 mμ could be due to purine- and pyrimidine-containing compounds formed from the degradation of nucleic acids.

In order to determine whether the effects of ribonuclease reported here were specific for the enzyme, some of the experiments were repeated using crystalline desoxyribonuclease (100 μgm/ml). The results indicated that up to 20 min of pretreatment, desoxyribonuclease had no effect on the absorption of Rb and on the staining by toluidine blue of excised mung bean roots.

SUMMARY

The effects of crystalline ribonuclease on Rb and phosphate absorption and on staining by toluidine blue of excised mung bean roots were investigated. The enzymic pretreatment increased the uptake of Rb in the absence of Ca and Mg. In the presence of Ca and Mg, treated roots absorbed markedly less Rb. Phosphate absorption was decreased both in the absence and presence of Ca by the ribonuclease treatment. Root cells pretreated with the enzyme showed less staining with toluidine blue than untreated cells. Desoxyribonuclease had no effect on Rb uptake and on staining by toluidine blue of excised roots. Ribonuclease solutions which were in contact with excised roots showed two absorption maxima in the ultraviolet, one at 250 to 260 mμ and the other at 290 to 300 mμ. These results have been interpreted to suggest the involvement of a ribonucleoprotein in ion absorption by mung bean roots.

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LITERATURE CITED

1. BRACHET, J. Effects of ribonuclease on the metabolism of living root-tip cells. Nature 174: 876–877. 1954.
2. HELLER, I. M. and KOPAC, M. J. Cytochemical reac-

tions of normal and starving *Amoeba proteus*. I. Localization of thiols, DNA, RNA and other organic phosphates. Exptl. Cell Research 8: 62–75. 1955.
3. KAUFMANN, B. P. and DAS, N. K. Production of mitotic abnormalities by ribonuclease. Proc. Nat. Acad. Sci., U. S. 40: 1052–1056. 1954.
4. LANSING, A. I. and ROSENTHAL, T. B. The relation between ribonucleic acid and ionic transport across the cell surface. Jour. Cellular Comp. Physiol. 40: 337–345. 1952.
5. LUNDEGÅRDH, H. Anion respiration. The experimental basis of a theory of absorption, transport and exudation of electrolytes by living cells and tissues. In: Active Transport and Secretion, R. Brown and J. F. Danielli, ed. Pp. 262–296. Academic Press Inc., New York 1954.
6. OVERSTREET, R. and JACOBSON, L. Mechanisms of ion absorption by roots. Ann. Rev. Plant Physiol. 3: 189–206. 1952.
7. TANADA, T. Effects of ultraviolet radiation and calcium and their interaction on salt absorption by excised mung bean roots. Plant Physiol. 30: 221–225. 1955.
8. VIETS, F. G., JR. Calcium and other polyvalent cations as accelerators of ion accumulation by excised barley roots. Plant Physiol. 19: 466–480. 1944.

KINETIN AND AUXIN ACTIVITY[1]

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Kinetin (6-furfurylaminopurine) has been shown to increase the rate of cell division in tobacco wound callus tissue when used in conjunction with 2 μgm/ml of indoleacetic acid (IAA) (2, 3). The present experiments were undertaken to determine the effect of kinetin on the responses of fragments of sunflower hypocotyl to IAA previously described (1).

The sunflower hypocotyl test for multiple auxin activity (1) was employed because this test demonstrates not only the stem-elongation inducing activity of an auxin but also its growth promoting (in the sense of increase in dry weight) and root initiating activity. All these activities have been shown to be correlated with auxin concentration, point of application of the auxin and position on the hypocotyl from which the fragment was excised. Sterile sunflower embryos were germinated on agar in darknes as described previously (1). When three days old they were removed and the 5-mm fragment immediately below the cotyledon (apical) and the fragment immediately above the root (basal) were removed. The fragments were transferred to sucrose mineral agar to which IAA and synthetic kinetin had been added before autoclaving in the concentrations shown in the tables. Stem elongation was studied using apical fragments placed upright with their apical ends in contact with the agar and cultured in complete darkness for 3 days. Weight increase and root production by apical fragments were studied by placing the fragment with the basal end on the agar and culturing it in darkness for 14 days. Weight increase and root production in the basal fragments were studied by placing the basal 5 mm with its apical end on the agar and culturing it for 14 days. At the end of the growth period, length, fresh and dry weights and number of roots on each fragment were determined. Average values obtained are shown in tables I, II and III. Ten replicates were used in each experiment. The means are subject to a standard error of ± 10 %. The following conclusions can be drawn from these figures:

1. The elongation induced in apical sunflower fragments by IAA (0.1 μgm/ml) applied to the apical end of the fragment was completely suppressed by 1.0

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TABLE I

AVERAGE LENGTH OF APICAL HYPOCOTYL FRAGMENT AFTER 3 DAYS' CULTURE WITH APICAL END ON SM AGAR ± IAA (0.1 μGM/ML) + KINETIN (MEANS OF 10 ESTIMATES)

IAA	KINETIN CONC (μGM/ML)			
	1.0	0.1	0.01	0
	mm	*mm*	*mm*	*mm*
Present	10.0	12.8	13.0	16.2
Absent	9.7	11.0	11.0	11.1

TABLE II

FRESH AND DRY WEIGHTS OF APICAL FRAGMENTS AFTER 14 DAYS' CULTURE WITH BASAL END ON SM AGAR ± IAA (1 μGM/ML) + KINETIN (MEANS OF 10 ESTIMATES)

IAA	KINETIN CONC (μGM/ML)							
	1.0		0.1		0.01		0	
	FRESH WT	DRY WT	FRESH WT	DRY WT	FRESH WT	DRY WT	FRESH WT	DRY WT
	mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
Present ...	228	14	208	14	259	16	245	13
Absent ...	99	6	121	7	59	3	53	3

TABLE III

AVERAGE NUMBER OF ROOTS ON BASAL FRAGMENTS AFTER 14 DAYS' CULTURE WITH APICAL END ON SM AGAR ± IAA (0.1 μGM/ML) + KINETIN (MEANS OF 10 ESTIMATES)

IAA	KINETIN CONC (μGM/ML)			
	1.0	0.1	0.01	0
Present	0	2.0	3.0	3.6
Absent	0	0	0.5	0

μgm/ml of kinetin and partly suppressed by 0.1 and 0.01 μgm/ml of kinetin.

2. In the absence of auxin, 1.0 μgm/ml of kinetin suppressed the elongation of such fragments on sucrose mineral agar.

3. The increase in fresh weight and dry weight induced in apical sunflower fragments cultured on sucrose mineral agar by 1 μgm/ml of IAA was not significantly changed by the presence of kinetin at a concentration of 1, 0.1 or 0.01 μgm/ml.

4. In the absence of auxin, 1.0 and 0.1 μgm/ml kinetin produced a significant increase in fresh and dry weight of these fragments which was about half that produced by 1 μgm/ml of IAA.

5. The initiation of adventitious roots induced by 0.1 μgm/ml of IAA applied to the apical end of basal sunflower fragments was inhibited completely by 1.0 μgm/ml of kinetin and partially by 0.1 μgm/ml kinetin.

6. In the absence of auxin, kinetin did not induce the initiation of adventitious roots in these fragments.

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LITERATURE CITED

1. DE ROPP, R. S. and MARKLEY, E. The correlation of different aspects of auxin action. Plant Physiol. 30: 210–214. 1955.
2. MILLER, C. O., SKOOG, F., VON SALTZA, M. H. and STRONG, F. M. Kinetin, a cell division factor from deoxyribonucleic acid. Jour. Amer. Chem. Soc. 77: 1392. 1955.
3. MILLER, C. O., SKOOG, F., OKUMURA, F. S., VON SALTZA, M. H. and STRONG, F. M. Structure and synthesis of kinetin. Jour. Amer. Chem. Soc. 77: 2662. 1955.

ANESTHETIZATION BY DI-ETHYL ETHER AND THE TRANSPORT OF FOLIAR APPLIED RADIOCALCIUM[1,2,3]

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The absence of downward transport of Ca^{45} in higher plants has been reported by Bledsoe (1), Norton (4), Martin (3), and others. It has been subsequently determined in our laboratory that there is little or no transport of Ca^{45} through graft unions of herbaceous plants involving reversed polarity (2). This has suggested the possible role of polarity in the movement of calcium. That polarity in plants might be temporarily suspended by mild anesthesia has been recorded by Went and Thimann (5).

Accordingly, $Ca^{45}Cl_2$ was applied dropwise to one of the two fully expanded primary leaves of the bean (*Phaseolus vulgaris*, var. Black Valentine). Plants with a Ca^{45}-treated primary leaf were then grouped as follows:

(a) The primary leaf treated with Ca^{45} was exposed for 96 hours in a 500-ml glass jar at room temperature to vapors from 25 ml of ether (diethyl).
(b) The leaf opposite the Ca^{45}-treated leaf was exposed to ether as in (a) above.
(c) The Ca^{45}-treated leaf was placed in a comparable glass jar with no ether.
(d) The leaf opposite the Ca^{45}-treated leaf was placed in a comparable glass jar with no ether.

Plants were harvested after the designated 96 hours of exposure and autoradiograms were prepared as described by Wittwer and Lundahl (6).

In contrast to previous experiments demonstrating the absence of downward transport of calcium in plants, exposure of the Ca^{45}-treated leaf to ether resulted in considerable transport of Ca^{45} out of the leaf and into other parts of the plant including the roots (fig 1). When the primary leaf opposite the Ca^{45}-treated leaf was subjected to ether vapors (group b) some basipetal transport of Ca^{45} was noted within the leaf itself. No transport, however, occurred out of the Ca^{45}-treated leaf when the opposite leaf was anesthetized. Likewise, no appreciable basipetal transport of Ca^{45} was evident from the site of application in either group (c) or (d). Under the conditions described the leaves subjected to ether vapors and to Ca^{45} exhibited no visible injury and no modification.

SUMMARY

The immobility and absence of downward transport of calcium in bean plants has been established with radiocalcium. Bean leaves treated with $Ca^{45}Cl_2$

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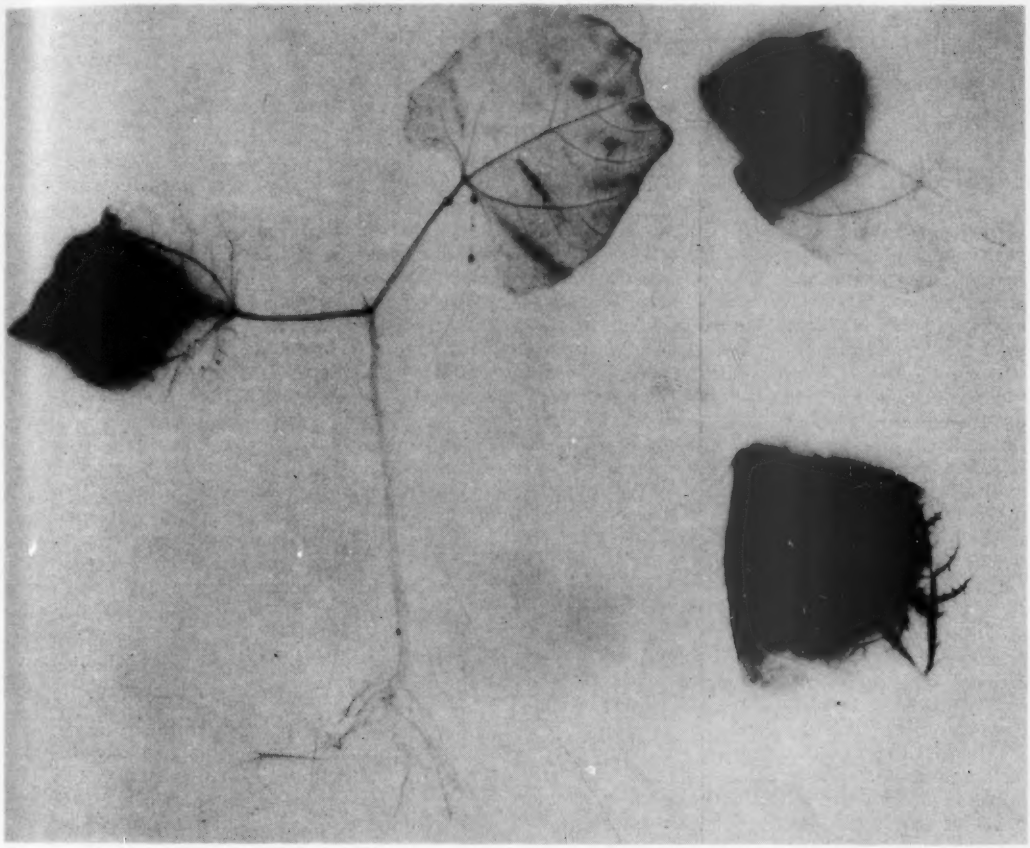

FIG. 1. Autoradiograms showing distribution of foliar applied Ca^{45} in the bean plant. (*Left*) Anesthetized Ca^{45}-treated leaf showing considerable transport out of the treated leaf; (*upper, right*) Ca^{45}-treated leaf opposite an anesthetized leaf showing very little transport; (*lower, right*) Ca^{45}-treated leaf with no basipetal transport of Ca^{45} from a plant receiving no anesthetization.

and subsequently anesthetized with di-ethyl ether transported considerable quantities of calcium from the site of application to all other parts of the plant as demonstrated by autoradiography.

LITERATURE CITED

1. BLEDSOE, R. W. Radiocalcium as an indicator of the necessity of calcium in the root environment. Paper presented before Amer. Soc. of Plant Physiol., Annual Meeting, Ithaca, New York, September 7 to 10, 1952.
2. BUKOVAC, M. J., TUKEY, H. B. and WITTWER, S. H. Transport of P^{32} and Ca^{45} in plants as influenced by graft unions and scion stock relationships. Abstr. of paper presented before the Conf. on Radioactive Isotopes in Agr., Michigan State Univ., East Lansing, Michigan, January 12 to 14, 1956.
3. MARTIN, D. C. The absorption and translocation of radiostrontium by the leaves, fruits, and roots of certain vegetable plants. Ph.D. thesis, Michigan State University, East Lansing, Michigan 1954.
4. NORTON, R. A. Nutrition of the strawberry with special reference to foliar absorption of radiophosphorus and calcium. Ph.D. thesis, Michigan State University, East Lansing, Michigan 1954.
5. WENT, F. W. and THIMANN, K. V. Phytohormones. P. 95. Macmillan, New York 1937.
6. WITTWER, S. H. and LUNDAHL, W. S. Autoradiography as an aid in determining the gross absorption and utilization of foliar applied nutrients. Plant Physiol. 26: 792–797. 1951.

EFFECT OF SPRAY RESIDUES AND OTHER CONTAMINANTS ON LEAF ANALYSIS[1,2]

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The problem of obtaining fruit- and nut-tree leaves free of surface contamination for mineral analysis has been of serious concern to workers in the field of plant nutrition. Several investigators (2, 5, 6, 7, 8) show that surface contamination may cause serious errors in iron, arsenic, and other constituents of fruit-tree leaves, and they stress the importance of its removal prior to chemical analysis for these elements. Bennett (2) and Jacobson (5) report that washing fruit-tree leaves in distilled water was not sufficient for the complete removal of surface contamination. Jacobson states that a thorough washing of the leaves in dilute acid is a prerequisite for valid quantitative analysis for iron. Lindner (6) reports that surface contamination on peach leaves near apple and pear orchards sprayed with lead arsenate may be sufficient to cause serious errors in analysis for arsenic. Smith et al (8) found that scrubbing both sides of leaves in 0.3 *N* HCl or neutral detergent was necessary for removal of surface contamination from citrus leaves. More recently, Mason (7) investigated several methods of cleaning apple leaves prior to chemical analysis and studied the effect of leaf washing on leaching of mineral constituents from the tissue.

Fungicidal sprays are necessary for the control of certain diseases of pecan, walnut, and other nut and fruit trees. In order to evaluate leaf-analysis data from fertilizer experiments, two leaf-washing tests were conducted to determine what effect spray residues have on the composition of pecan leaves; whether mineral elements such as Cu and Zn are absorbed from the spray mixture; and whether spray residues can be effectively removed from the leaves without leaching mineral elements from the tissues.

In the first experiment, leaf samples were collected August 17, 1951, from several sprayed and unsprayed 40-year-old pecan trees of the Delmas variety in a scab-control experiment at Winona, Texas. Four uniform samples of 20 leaves each were collected from 5 trees which had been previously sprayed with Bordeaux mixture and Ziram (zinc dimethyldithiocarbamate), and a like number from unsprayed trees, the third leaf from the terminal end of each shoot being taken for the samples.

The fresh green leaves were washed first in running tap water, both sides of each leaflet being scrubbed with a cotton swab, then for 1 minute in 0.5 % HCl (by volume) or in 1 % HCl, twice in distilled water, and finally in Cu- and Zn-free water. Aliquots of the various wash solutions were retained for analysis for Cu and Zn.

The second experiment was set up to study further the effectiveness of washing and other aspects of spray residue contamination on pecan leaves. Two sets of pecan leaf samples were collected at Robson, Louisiana, September 2, 1953, from twelve 25-year-old Schley trees which had been sprayed with Cu- or Zn-containing materials. One set of 4 samples of 30 leaves each were obtained from 6 trees which had been sprayed with 4 applications of Bordeaux mixture, and a like number of samples from 6 trees which had received 4 spray applications of Zineb (zinc ethylenebisdithiocarbamate) on the dates when the Bordeaux spray was applied. Leaves in the 4 samples from trees given each spray treatment were treated as follows: 1) not washed; 2) scrubbed on both sides under running tap water and rinsed in distilled water and in Cu- and Zn-free water; 3) scrubbed and washed first under running tap water and then 1 minute in 0.5 % HCl solution and rinsed as in 2); 4) treated the same as in 3) except that 1 % HCl was substituted for 0.5 % HCl.

After drying, the leaflets in both experiments were stripped from the rachises and ground in a Wiley laboratory mill equipped with a nickel-plated 20-mesh sieve. The leaves from the first experiment were analyzed for Cu and Zn, while those from the second experiment were analyzed for nitrogen and several mineral constituents. Standard methods (1) were used for the determination of N, P, Mg, Mn, and Fe, while K, Ca, and Na were determined by flame photometry. Modifications of the dithizone method for Zn (4) and of the dithiocarbamate method for Cu (3) were used in analysis for these elements.

Analyses of the wash solutions from Delmas pecan leaves in the first experiment showed that very little Cu or Zn was removed by the second washing of leaves with distilled water after they had been washed with acid, whereas a relatively high amount was removed by the subsequent water washings from leaves washed only with water. The Cu and Zn analysis of the washed leaves showed that water alone was not sufficient to remove the Cu spray residue, and that the HCl solution was more effective at 1 % than at 0.5 % concentration. Although the Cu found in the sprayed leaves after washing with 1 % HCl was probably not all internal, it is assumed that most of the difference between 24 ppm Cu found in the unsprayed leaves and 61 ppm Cu found in the acid-washed sprayed leaves was due to absorption of Cu from the Bordeaux spray.

The data in table I indicate that the Schley leaves from the second experiment absorbed Cu from the

[1] Received revised manuscript March 9, 1956.

[2] This paper was presented at the Plant Physiology section of the 52nd Annual Convention of Southern Agricultural Workers in Louisville, Kentucky on February 7, 1955.

TABLE I

CHEMICAL CONTENTS OF WASHED AND UNWASHED SCHLEY PECAN LEAVES (OVEN-DRY BASIS)

KIND OF SAMPLE *	%						PPM				
	ASH	N	P	K	Ca	Mg	Mn	Fe	Cu	Zn	Na
Leaves Sprayed with Zineb											
Unwashed	8.94	2.06	0.130	0.740	2.12	0.540	180	118	62	360	250
Washed with H_2O	8.84	2.12	0.130	0.740	2.12	0.546	180	90	48	125	240
Washed with 0.5 % HCl	8.83	2.18	0.130	0.735	2.11	0.552	180	70	25	96	230
Washed with 1 % HCl	8.77	2.13	0.130	0.745	2.12	0.540	180	70	24	74	240
Leaves Sprayed with Bordeaux											
Unwashed	9.66	2.04	0.125	0.740	2.23	0.540	136	155	1525	88	165
Washed with H_2O	9.22	2.12	0.130	0.740	2.21	0.556	136	95	950	60	175
Washed with 0.5 % HCl	9.17	2.15	0.128	0.745	2.22	0.552	136	80	260	50	195
Washed with 1 % HCl	9.15	2.08	0.128	0.740	2.22	0.540	136	75	195	44	180

* All washed-leaf treatments include complete washing procedure.

Bordeaux spray and Zn from the Zineb spray. Similar results were obtained by Smith et al (8), who concluded that citrus leaves previously sprayed with materials containing Fe, Cu, and Zn should not be used for diagnostic purposes for those particular elements because of apparent absorption.

The washing procedures had very little leaching effect on the nitrogen and mineral constituents commonly determined in leaf analysis, i.e. N, P, K, Ca, Mg, Mn, and Na. This was clearly demonstrated by the work of Smith et al (8) and Mason (7), who showed that leaching of elements by various leaf-washing procedures was negligible when the immersion time was limited. The present results, together with a large number of analyses of other washed and unwashed pecan, almond, filbert, and walnut leaves from unsprayed trees made during the past few years, show that Fe is the only element which was seriously affected in quantity by washing the leaves prior to analysis, in most cases being several times higher in samples of unwashed leaves because of surface contamination with dust. Washing did not appreciably alter the Ca content in the leaves sprayed with Bordeaux mixture, the accumulation of Ca in the residue from four applications of the spray not being enough to be measured in the analysis.

SUMMARY AND CONCLUSIONS

A series of leaf samples from unsprayed Delmas pecan trees and from similar trees sprayed with Bordeaux mixture and Ziram, and two series of leaf samples from Schley pecan trees which had been sprayed with Bordeaux mixture or with Zineb were washed with water alone, with 0.5 % HCl or with 1 % HCl solution, and then analyzed for several elements to determine the effectiveness of the washing procedure in the removal of spray residues and other surface contamination.

Spray residues and other contaminants were effectively reduced by washing with 1 % HCl solution and rinsing twice with distilled water, but a thorough washing with water was found to be inadequate. Results show clearly that there was no appreciable leaching of the chemical constituents of the leaf as a result of the washing procedures used. Pecan leaves apparently absorb copper and zinc from spray solutions containing them, and this fact must be considered in evaluating analytical data for these elements even though the leaves are thoroughly washed prior to analysis.

Nut-tree leaves generally carry enough iron as surface dust contamination to seriously alter the analysis for iron. The same may be true for other elements in some localities, and therefore it should be determined whether such contamination occurs before making analysis for any of the mineral elements. Also, the residues of Bordeaux mixture and Ziram and Zineb sprays contain enough copper and zinc to alter the chemical results for these elements.

LITERATURE CITED

1. ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS. Official and Tentative Methods of Analysis, 5th ed. Pp. 1–757. Assoc. Offic. Agr. Chem., Washington, D. C. 1940.
2. BENNETT, J. P. Iron in leaves. Soil Sci. 60: 91–105. 1945.
3. COULSON, E. J. Report on copper. Jour. Assoc. Off. Agr. Chem. 20: 178–188. 1937.
4. HALE, COWLING and MILLER, E. J. Determination of small amounts of zinc in plants. Jour. Ind. Eng. Chem. 13: 145–149. 1941.
5. JACOBSON, L. Iron in the leaves and chloroplasts of some plants in relation to their chlorophyll content. Plant Physiol. 20: 233–245. 1945.
6. LINDNER, R. C. Arsenic injury of peach trees. Proc. Amer. Soc. Hort. Sci. 42: 275–279. 1943.
7. MASON, A. C. The cleaning of leaves prior to analysis. Ann. Report East Malling Research Sta., Kent 1951–52: 104–107. 1953.
8. SMITH, P. F., REUTHER, W. and SPECHT, A. W. Mineral composition of chlorotic orange leaves and some observations on the relation of sample preparation technique to the interpretation of results. Plant Physiol. 25: 496–506. 1950.

The American Society of Plant Physiologists on the Formation of Sections: The Executive Committee of our Society directed that a note be drafted by a special committee pointing out for our members at large the conditions regulating section formation and the advantages which can accrue from such area groups. These are outlined for general information.

Constitutional Provision: Article IV of the Constitution relative to the formation of "Local, State, and Regional Sections" states:

1. Local, state, and regional sections of this organization may be formed upon the petition of 10 members resident in the locality, state, or region, providing that such (local,) * state, or regional organization is approved by a majority of the members voting on the petition.

2. Such sections shall have local autonomy in regard to their manner of organization, the holding of meetings, and other activities, providing that none of the local regulations violate any provisions of the Constitution.

3. Any section which fails to maintain at least 10 active members is automatically discontinued.

Present Sections: At present, there are four regional sections and one local section. The former encompass the Northeastern, Southern, Western, and Midwestern areas and are so designated. The Washington Section is delimited to the Washington, D. C., area.

Recent Section Changes: At an earlier date, there existed sections of local or state area status including the Minnesota, Purdue, and Illinois Sections. The Minnesota group became inactive. The Purdue and Illinois groups requested recognition as a unified section to be called the Midwestern Section. This union was favorably recognized by the A.S.P.P. Executive Committee to include these two groups as well as members from Minnesota and other states in the region. This new status was approved as furthering a more comprehensive participation and a more active and effective area meeting. The local Illinois, Purdue, and Minnesota Sections were individually dissolved.

Permission was granted the New England Section to change its name to the Northeastern Section. At the request of members in New York State, the possibility and desirability of a strong Middle Atlantic States Section has been considered. Until such a section is formed by members within this area, the Northeastern Section may feel free to invite physiologists outside of New England.

* Committee insert.

Section Activities: During the course of the last year, the Illinois and Purdue groups held a joint meeting prior to the merger as the Midwestern Section. Invitational papers were read and round-table discussions were held on various phases of physiology.

The Southern Section met with the Association of Southern Agricultural Workers, in Louisville, Kentucky. Two symposia were held and two sessions were devoted to submitted papers. Attendance was widespread and large. This activity has been important for some years. An annual meeting for 1956 has been projected.

The Western Section held its annual meeting, as usual for many years, with the Pacific Division of the A.A.A.S. The program included two symposia and four sessions of submitted papers. Attendance is always large, usually including our own members as well as numerous participants from among other botanists. The symposia are generally jointly sponsored with members of the Botanical Society and frequently with members of the Western Society of Soil Science.

Retrospection and Contemplation: All information and observation leads to the conclusion that section formation and active participation therein has been and can be of pleasure and benefit to our members. Meetings are held at times which do not conflict with or detract from our annual national meetings. The summer sessions of the Western Section with the Pacific Division of the A.A.A.S. (with which we are affiliated) as sponsor are particularly effective. These meetings serve those who cannot regularly attend the national fall sessions under the A.I.B.S. Much has been and can be accomplished at these area sessions.

The Executive Committee of our Society has recognized the inherent advantages of section association and participation. At its last session it went on record as encouraging section formation and meetings at times when additional discussion would be of benefit to our members. Members at large are urged to attend and participate actively in these sectional meetings and to invite and foster attendance by younger plant physiologists and others who as yet are not members of our Society. In this way it is hoped that we may enhance the effectiveness of our organization in encouraging and promoting "the growth and development of plant physiology as a pure and applied phase of botanical science" and "the general welfare and good fellowship of plant physiologists."—Committee, A. S. Crafts, H. A. Borthwick, T. C. Broyer.